From Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

THE KYNURENINE PATHWAY IN BODY AND BRAIN - RELATION WITH PHYSICAL EXERCISE AND MENTAL HEALTH

Ada Trepci

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The kynurenine pathway in body and brain - relation with physical exercise and mental health
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By

Ada Trepci

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Principal Supervisor:
Professor Sophie Erhardt
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Neuropsychoimmunology

Co-supervisor(s):
Dr. Kristian Sandberg
Uppsala University
Department of Medicinal Chemistry
Science for Life Laboratory

Professor Tomas Venckunas
Lithuanian Sports University
Institute of Sport Science and Innovations

Opponent:
MD, PhD, DSc(med), Professor Gregers Wegener
Aarhus University, Denmark
Department of Clinical Medicine
Translational Neuropsychiatry Unit

Examination Board:
Associate Professor Mia Lindskog
Uppsala University
Department of Neuroscience
Division of Medical Neurobiology

Professor Bo Runeson
Karolinska Institutet
Department of Clinical Neuroscience
Division of Centre for Psychiatry Research

Associate Professor Daniel Globisch
Uppsala University
Department of Chemistry - BMC
Division of Analytical Chemistry

To my parents

KEYWORDS

The shape of word cloud is a face mask, chosen as representative symbol of COVID-19 pandemic, period during which this thesis was finalized.

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ABSTRACT

The kynurenine pathway of tryptophan degradation is the focus in a variety of research fields and several of the metabolites produced along the pathway are suggested as future biomarkers. Growing interest in a better understanding of the physiological and the pathophysiological importance of kynurenine pathway metabolites is followed by the need for a robust, sensitive method that can quantify multiple metabolites simultaneously. In the present thesis, the first aim was to develop and validate robust and sensitive methods for the analysis of kynurenine pathway metabolites in body and brain. Our method thus allows for simultaneous quantification of low concentrations of tryptophan, kynurenine, kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HANA), quinolinic acid (QUIN), picolinic acid (PIC), nicotinic acid (NA) and nicotinamide (NAA) in human cerebrospinal fluid (CSF) and plasma. In addition, our new method clearly separates the peaks of the isomers, PIC and NA. Moreover, metabolites of the kynurenine pathway have been linked to the pathophysiology of bipolar disorder and suicide. In this thesis, we thus analyzed the concentrations of kynurenine pathway metabolites in the CSF of suicide attempters and patients with bipolar disorder. All patients were stratified into groups according to their history of suicide behavior. As previously reported, we found a reduced CSF PIC/QUIN ratio in suicide attempters. Furthermore, patients with bipolar disorder and a history of suicide behavior had lower CSF PIC concentrations than those without, giving further support for the hypothesis that low CSF PIC is a predictor of suicidality vulnerability. A negative correlation between an ACMSD genetic variant and the CSF PIC/QUIN ratio in patients with bipolar disorder with a history of suicide behavior was discovered, suggesting that polymorphism in ACMSD is linked to excess QUIN formation at the expense of PIC. In patients with bipolar disorder, we also found elevated CSF kynurenine/tryptophan ratio, KYNA, and PIC concentrations clearly demonstrating induction of the kynurenine pathway in these patients.

In the process of developing biomarkers, it is also important to understand if and how daily activity, such as physical exercise affects their concentration. The majority of exercise studies investigating kynurenines published so far have focused on a few kynurenine metabolites, and most often not taken into consideration the exercise habit of the participants that might affect the results. In the last part of this thesis, we thus investigated how different types of exercise influence central and peripheral levels of kynurenine pathway metabolites. Specifically, we investigated how one bout of sprint interval exercise (SIE) affects plasma kynurenine pathway metabolites of healthy subjects belonging to different age groups. In detail, the SIE session consisted of 6 repetitions of 30-seconds all-out cycling with 4-minutes passive recovery periods

in between. Moreover, in another cohort of healthy subjects with a background of different exercise habits, we investigated how aerobic exercise affected kynurenine pathway metabolites and immune markers in plasma and CSF. In this study, participants were divided into two groups based on their existing level of physical activity habits. One group performed an acute intense exercise program for four days (two days of 30 minutes high-intensity interval training, and two days of running for at least 60 minutes) and the second training group performed 30 minutes of running, three times weekly, for 4 weeks.

The results clearly show that both types of exercise tested (aerobic and anaerobic) influence kynurenine pathway metabolites. We found that plasma levels of kynurenine increased 1 hour after the SIE session in healthy elderly subjects, while levels of KYNA increased after 24 hours. We further found that acute exercise intervention, increased the CSF levels of KYNA, 3HK, and PIC, while tryptophan and kynurenine remained unchanged. Furthermore, in the training group, the CSF kynurenine/tryptophan ratio increased. Tryptophan and kynurenine, on the other hand, decreased significantly after acute exercise, while KYNA, 3-HK, QUIN, and PIC in the plasma did not change in any of the groups. Some immune markers showed a tendency towards an increase in both plasma and CSF but were not statistically significant. In plasma, kynurenine and PIC levels were correlated with aberrant immune markers profile. While in CSF, kynurenine and QUIN levels, and also the ratio of kynurenine/tryptophan were associated with immune protein marker profiles. One important finding was the positive correlation between PIC levels in plasma and CSF.

In the present thesis, we have developed a novel method for the simultaneous quantification of kynurenine pathway metabolites. We also confirm that the brain kynurenine pathway in patients with bipolar disorder is activated as well as we show data supporting that low PIC might indicate vulnerability for suicidal behavior. Furthermore, we confirm that both central and peripheral kynurenine pathway metabolites are affected by physical exercise.

LIST OF SCIENTIFIC PAPERS

I. Lilly Schwieler, **Ada Trepci**, Stanislaw Krzyzanowski, Sigurd Hermansson, Mathias Granqvist, Fredrik Piehl, Tomas Venckunas, Marius Brazaitis, Sigitas Kamandulis, Daniel Lindqvist, A. Daniel Jones, Sophie Erhardt, Lena Brundin. **A novel, robust method for quantification of multiple kynurenines pathway metabolites in the cerebrospinal fluid.** Bioanalysis, 12(6):379-392, 2020

II. **Ada Trepci**, Carl M. Sellgren, Erik Pålsson, Lena Brundin, Neda Khanlarkhani, Lilly Schwieler, Mikael Landén, Sophie Erhardt. **Central levels of tryptophan metabolites in subjects with bipolar disorder.** European Neuropsychopharmacology, 43:52-62, 2021

III. **Ada Trepci**, Sophie Imbeault, Victoria L. Wyckelsma, Håkan Westerblad, Sigurd Hermansson, Daniel C. Andersson, Fredrik Piehl, Tomas Venckunas, Marius Brazaitis, Sigitas Kamandulis, Lena Brundin, Sophie Erhardt, Lilly Schwieler. **Quantification of plasma kynurenine metabolites following one bout of sprint interval exercise.** International Journal of Tryptophan Research, 13:1178646920978241, 2020

IV. Josef Isung, Mathias Granqvist, **Ada Trepci**, Jesse Huang, Lilly Schwieler, Marie Kierkegaard, Sophie Erhardt, Jussi Jokinen, Fredrik Piehl. **Differential effects on blood and cerebrospinal fluid immune protein markers and kynurenine pathway metabolites from aerobic physical exercise in healthy subjetcs**. Scientific Reports, 11(1):1669, 2021

CONTENTS

LIST OF ABBREVIATIONS

3-HAAO	3-hydroxianthralinate 3,4-dioxigenase
3-HANA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenine
2-MCE	2-mercaptoethanol
α7nACh	α-7 nicotinic acetylcholine
AA	Anthranilic acid
ACMS	2-amino 3-carboxymuconic semialdehyde
ACMSD	2-amino 3-carboxymuconate 6-semialdehyde decarboxylase
AhR	Arylhydrocarbon receptor
AMPA	a-amino-3-hydroxy-5-methylisoxazole-4-propionate
BBB	Blood brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ES+	Positive electrospray ionization mass spectrometry
FDA	Food and Drug Administration
FDR	False discovery rate
GABA	γ-aminobutyric acid
GDPR	General Data Protection Regulation
GPR35	G protein-coupled receptor
GWAS	Genome-wide association studies
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentrationn
IDO	Indolamine-pyrrole 2, 3-dioxygenase
IFN	Interferon
IL	Interleukin

IQR	Inter Quartile range
IS	Internal standard
KaSP	Karolinska Schizophrenia Project
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxygenase
KYNA	Kynurenic acid
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPS	Lipopolysaccharide
MADRS	Montgomery-Åsberg Depression Rating Scale
MCP-1	Monocyte chemotactic protein-1
MINI	Mini International Neuropsychiatric Interview
MMD	Major depressive disorder
MMP	Matrix metalloproteinase
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NA	Nicotinic acid
NAA	Nicotinamide
NAD^+	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NPX	Normalized protein expression
OPA	O-phthaldialdehyde
PEA	Proximity extension assay
PGC 1α1	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PIC	Picolinic acid
QPRT	Quinolinate phosphoribosyltransferase
QUIN	Quinolinic acid
RPE	Rating of Perceived Exertion
RSD	Relative standard deviation
SBP	St. Göran Bipolar Project

SCID	Structured Clinical Interview
SD	Standard deviation
SIE	Sprint interval exercise
S/N	Signal to noise ratio
SNP	Single nucleotide polymorphism
TBE	Tick-borne encephalitis
TDO2	Tryptophan 2,3 dioxygenase
TIC	Total ion current
TNF	Tumor necrosis factor
UPLC-MS/MS	Ultrahigh-performance liquid chromatography / tandem mass spectrometry
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XA	Xanthurenic acid
YMRS	Young Mania Rating Scale

1 INTRODUCTION

1.1 The kynurenine pathway of tryptophan degradation

In mammals, tryptophan can be catabolized into serotonin and melatonin as well as via the kynurenine pathway. Around 90-95% is degraded through the latter pathway and is thus, considered as the primary route of degradation from a quantitative perspective (Leklem, 1971; Guidetti et al., 1995; Schwarcz & Stone, 2017) (Figure 1).

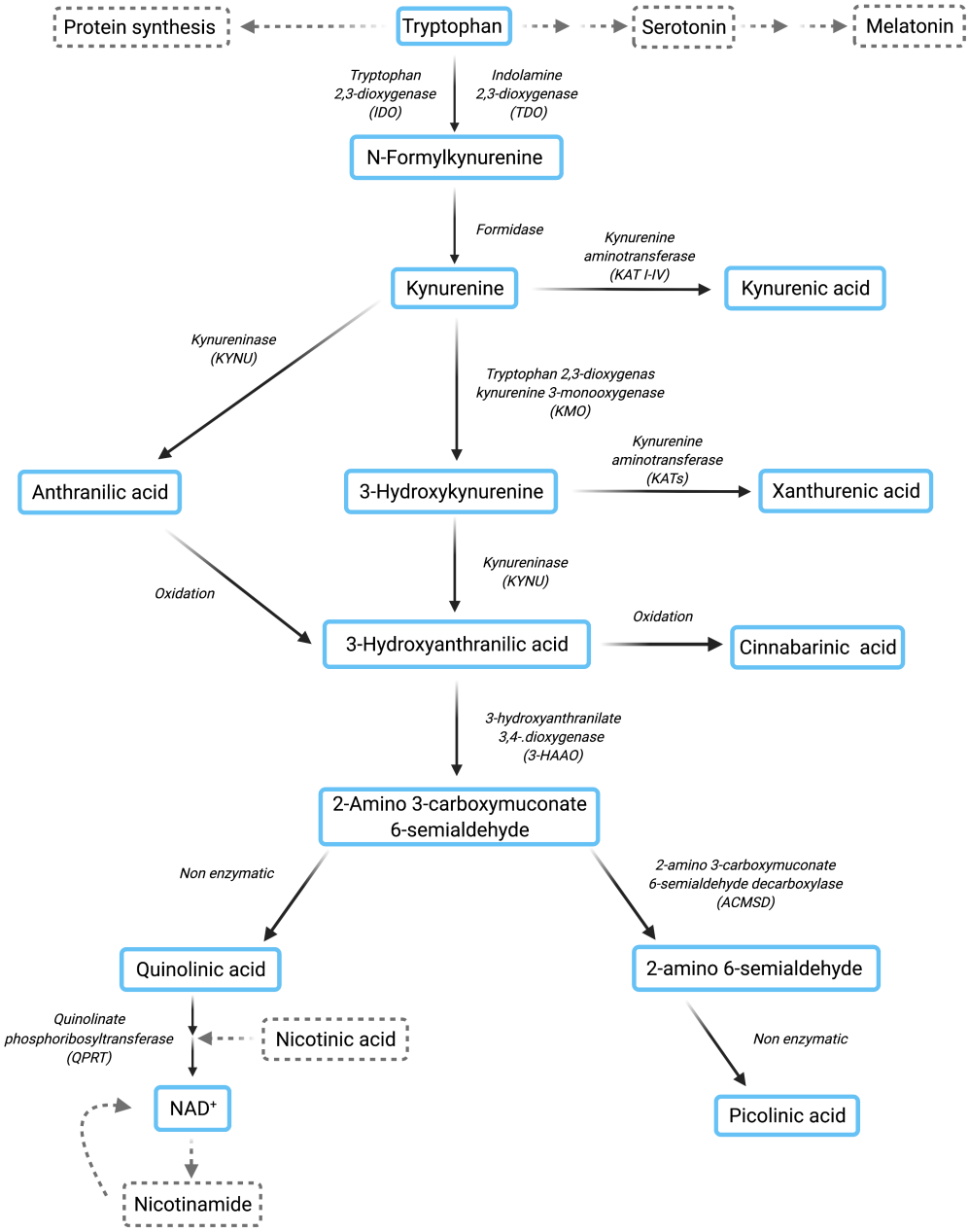

Figure 1. The kynurenine pathway of tryptophan degradation

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The kynurenine pathway of tryptophan degradation has gained a lot of attention during the last decades because first, the kynurenine pathway produces the nicotinamide adenine dinucleotide (NAD^+) which is the energy for the cells, and plays a crucial role in the wellbeing of humans, and second, several neuroactive metabolites are produced along kynurenine pathway.

Kynurenine pathway begins with the hydroxylation of tryptophan into N-formylkynurenine by enzymes: indolamine 2,3 dioxygenase 1 (IDO1) (Hayaishi, 1976), indolamine 2,3 dioxygenase 2 (IDO2) (Ball et al., 2009) and/or tryptophan 2,3 dioxygenase (TDO2) (Hayaishi et al., 1957; Hayaishi, 1993). It continues with the hydrolyzation of N-formylkynurenine into kynurenine by the kynurenine formamidase (Knox & Mehler, 1950). Then kynurenine is further catabolized into three products, by different enzymes:

1. Kynureninase, hereby producing anthranilic acid (AA).
2. Kynurenine 3 monooxygenase (KMO), producing 3-hydroxykynurenine (3-HK), a free radical generator.
3. By irreversible transformation through four isoforms of enzymes kynurenine aminotransferase (KATs: KAT: I, II, III, IV), producing the end-metabolite kynurenic acid (KYNA) (Han et al., 2010).

Both 3-HK and AA are catabolized to 3-hydroxyanthranilic acid (3-HANA), through the enzyme kynureninase or via non-enzymatic oxidation, respectively. 3-HK might also be catabolized to xanthurenic acid (XA) through KATs. Subsequently 3-HANA, through the enzyme 3-hydroxyanthralinate 3,4-dioxigenase (3-HAAO) is converted to 2-amino 3-carboxymuconic semialdehyde (ACMS) or through non-enzymatic oxidation to cinnabarinic acid (Christen et al., 1992). ACMS serves as substrate for 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase (ACMSD) and 2-aminomuconic 6-semialdehyde is generated, which spontaneously converts to picolinic acid (PIC) (Mehler, 1956). When the enzyme ACMSD is saturated, missing, or less active, the spontaneous formation of quinolinic acid (QUIN) from ACMS is enhanced. Finally, QUIN is catabolized by quinolinate phosphoribosyltransferase, leading to NAD^+. The production of NAD^+ via the kynurenine pathway is inversely proportional to the activity of the enzyme ACMSD (Shibata & Fukuwatari, 2015). The kynurenine pathway of tryptophan degradation is the essential route for *de novo* synthesis of NAD^+ in mammalian cells. However, in cases of diets poor in tryptophan, the *de novo* NAD^+ synthesis decreases, and alternatively, NAD^+ is recycled by nicotinic acid (NA) and nicotinamide (NAA), well known as “salvageable precursors” (Bogan & Brenner, 2008; Braidy et al., 2019).

1.1.1 Kynurenines in body and brain

All kynurenine pathway enzymes are present both in body and brain, however, generated metabolites are tissue and cell specific (Platten et al., 2019). Notably, the relative abundance of the metabolites produced along the pathway, in body and in brain, is thought to be regulated at various interdependent levels (Badawy, 2017). Thus, the concentration of kynurenine metabolites in the periphery may affect brain levels of some of the kynurenine metabolites.

The majority of tryptophan in the periphery is metabolized in the liver but tryptophan is also transported into the brain through the blood-brain barrier (BBB) by the large neutral amino acid transporter. Tryptophan competes with other large amino acids, such as leucine, isoleucine, tyrosine, methionine, and valine when entering the brain. Thus, the amount of tryptophan entering the brain depends on plasma tryptophan concentration as well as its relative concentration compared to the concentrations of other large amino acids (Fernstrom, 1983).

Kynurenine can be transported through the neutral amino acid transporter over the BBB, but can also be produced locally in the brain (Gál & Sherman, 1980; Ramos-Chávez et al., 2018). In physiological conditions, periphery provides approximately 60% of the kynurenine present in the brain (Gál & Sherman, 1980; Fukui et al., 1991). In the brain, kynurenine is produced by neurons, astrocytes, microglia, microvascular endothelial cells, and macrophages (Guillemin et al., 2007). In the periphery, kynurenine is produced in the lungs, placenta, stomach, intestines, spleen, kidney and liver (Stone, 1993; King & Thomas, 2007). In low micromolar concentration (Opitz et al., 2011), kynurenine has an affinity for the aryl hydrocarbon receptors (AhR). AhR is expressed in peripheral organs, such as adipose tissue, lungs, liver, and also in the brain. Immune regulation, cell differentiation and tumorigenesis are affected by the AhR (Mezrich et al., 2010; Opitz et al., 2011; Yamamoto et al., 2019).

KYNA is produced by a variety of peripheral cells and organs, such as the liver, kidney, and heart, and is detected in multiple biological fluids, including urine, blood, milk, cerebrospinal fluid (CSF), saliva, gastric juice, amniotic fluid, pancreatic juice and bile (Milart et al., 1999; Kuc et al., 2006; Paluszkiewicz et al., 2009; Milart et al., 2019). KYNA has a polar structure and does not easily pass the BBB. One rodent study showed that in case of exceedingly high levels of KYNA in the periphery and in case the BBB is compromised, KYNA can enter the brain (Scharfman et al., 2000). However, the influx rate of which KYNA enters the brain from the periphery is very low and is calculated to be 0.0023 nmol/h/g (Fukui et al., 1991). In the brain, KYNA is mostly produced by astrocytes (Kiss et al., 2003; Rossi et al., 2008). KYNA is known for having neuroprotective properties (Moroni, 1999). KYNA binds to a wide range of

different receptors. When KYNA is present in low concentrations (IC_{50}: 8-15 μM), it acts as an antagonist at the glycine binding site of the N-methyl-D-aspartate (NMDA) receptor (Birch et al., 1988) and when KYNA is present in high concentrations (IC_{50}: 0.2-0-5 mM) it acts as an antagonist at the glutamate recognition site of the same receptor (Kessler et al., 1989). KYNA shows antagonistic properties also to the kainate and a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors (Perkins & Stone, 1982; Kessler et al., 1989). Furthermore, KYNA is suggested to be a non-competitive inhibitor of α-7 nicotinic acetylcholine (α7nACh) receptor (IC_{50}: 7 μM) (Hilmas et al., 2001), although some controversies regarding this remain (Stone, 2020). Also, KYNA shows agonistic properties to other receptors, such as AhR (DiNatale et al., 2010) and G protein-coupled receptor 35 (GPR35) (Wang et al., 2006).

In the periphery, QUIN is produced in several organs and can be detected in biological fluids such as urine, blood, and CSF. Central QUIN originates from local sources in the brain; mainly microglial cells and macrophages that have crossed the BBB (Guillemin, 2012). QUIN is an excitotoxic NMDA receptor agonist (Stone & Perkins, 1981; Schwarcz et al., 2012), known to be neurotoxic mainly through two mechanisms: 1. increasing glutamate release and 2. inhibiting glutamate uptake (Guillemin et al., 2001). Rodent studies showed that the affinity of QUIN to NMDA receptor is affected also by the endogenous iron levels (Šťastny et al., 1999). The half-life of QUIN is relatively short, around 20 minutes (Bryleva & Brundin, 2017).

PIC can be found in human CSF and plasma, as well as in human milk, pancreatic juice and intestinal homogenates. The ability of PIC to chelate copper, iron and other metals is its most well-known physiological function (Grant et al., 2009). PIC in the brain possesses neuroactive properties. PIC can act as an agonist of strychnine-sensitive glycine receptors in spinal neurons, in addition to having a protective effect in the brain (Tonohiro et al., 1990; 1997). Thus, PIC is thought to mitigate the neurotoxicity caused by QUIN by inhibiting glutamate release from the presynaptic neurons (Vrooman et al., 1993; Beninger et al., 1994). While neurotoxicity caused by QUIN might be blocked by PIC, neuroexcitatory effects of QUIN are not affected (Jhamandas et al., 2000).

1.1.2 Immune-induced activation of the kynurenine pathway

The kynurenine pathway of tryptophan degradation is strongly influenced by the immune system and can be activated by a variety of inflammatory parameters. For example, the rate-limiting enzyme of the pathway IDO1 can be triggered by tumor necrosis factor (TNF)-α, TNF-β, interferon (INF)-α, INF-β, and INF-γ (Guillemin et al., 2001; Bergström et al., 2002; Wichers & Maes, 2004; King & Thomas, 2007; Munn & Mellor, 2016). IDO1 is also induced by other cytokines, such as interleukins (IL), for example, IL-1α, IL-6, and IL-10 (Babcock & Carlin, 2000, Yanagawa et al., 2009; Hayley, 2011). TDO2 is instead induced by IL-1β (Urata et al., 2014; Sellgren et al., 2016) and is additionally regulated by corticosteroids (Campbell et al., 2014). Another important enzyme of the pathway, KMO, which leads to the excitatory branch of the pathway is activated by immune markers (Garrison et al., 2018; Lu et al., 2020). For instance, KMO expression is elevated by IL-1β (Zunszain et al., 2012).

Several experimental studies have shown that challenging the immune system induces the kynurenine pathway. Thus, dual lipopolysaccharide (LPS) injections of 0.83 mg/ kg increase the levels of kynurenine and KYNA in the mouse brain and serum, as well as QUIN in the brain (Larsson et al., 2016; Tufvesson-Alm et al., 2020). Levels of KYNA are also increased in mice brain after a single dose of 2 mg/kg of LPS but not from the dose of 0.32mg/ kg and 0.8 mg/kg (Tao et al., 2020). Furthermore, LPS induces IDO1, resulting in decreased tryptophan and increased kynurenine levels in mouse plasma (O'connor et al., 2009). Following LPS treatment, KMO expression rises along with an increase in brain IL-6 (Connor et al., 2008; Molteni et al., 2013). LPS can also increase the levels of 3-HK in the mouse brain, both in the cortex and hippocampus (Tao et al., 2020).

Clinical studies have shown that the kynurenine pathway is activated in several infectious diseases. Thus, patients with tick-borne encephalitis (TBE) have elevated CSF levels of KYNA and KYNA correlates with the severity of the disease (Holtze et al., 2012). On the other hand, children (median age 142 months old) with TBE do not show changes in levels of KYNA and kynurenine in the CSF (Wickström et al., 2021). Elevated levels of CSF KYNA were also seen in subjects with herpes simples virus type 1 encephalitis (Atlas et al., 2013) or human immunodeficiency virus type (HIV) (Atlas et al., 2007). Moreover, IDO1 activity is elevated in the plasma of subjects with HIV (Favre et al., 2010). In addition, children with malaria have elevated levels of kynurenine and KYNA in the CSF (Holmberg et al., 2017).

1.2 Analysis of kynurenines and challenges

Metabolites produced along the kynurenine pathway are gaining more and more attention and are suggested to have crucial roles both in health and in diseases, such as cancer, infections, neurodegenerative diseases as well as neurological, cardiovascular, immunological, and psychiatric disorders (Widner et al., 2000; Atlas et al., 2007; Olsson et al., 2010; Linderholm et al., 2012; Schwarcz et al., 2012; Polyzos & Ketelhuth, 2015; Rajda et al., 2015; Schwieler et al., 2016; Erhardt et al., 2017; Majlath et al., 2018; Platten et al., 2019).

Most studies so far have focused on a limited number of the metabolites in human CSF and plasma. In order to get a more comprehensive picture of the regulation and activity of the kynurenine pathway, both in physiological and pathophysiological conditions, it is important to simultaneously quantify several kynurenine metabolites using the same method. When establishing such a method, there are a few things to consider and challenges that arise along the way.

First, the endogenous concentration of the kynurenine metabolites ranges from nanomolar to micromolar (Linderholm et al., 2012; Schwarcz et al., 2012; Kegel et al., 2014; Brundin et al., 2016). In addition, the concentration of a specific metabolite is also tissue- and/or biological fluid-specific as well as health- or disease-state-specific. For example, tryptophan in the CSF and plasma is present at micromolar concentrations while kynurenine in CSF is present at nanomolar concentrations but in plasma is in the micromolar range. The other metabolites, such as KYNA, 3-HK, QUIN, 3-HANA, XA, PIC, NA, NAA are found in nanomolar concentrations both in CSF and in plasma. Thus, for some metabolites, a lower sensitivity is needed for detection, while for others, a higher sensitivity should be maintained.

Second, the chemical structures of the kynurenine metabolites are closely related, which makes it impossible to use antibody-based detection methods. For instance, two of the kynurenine metabolites, PIC and NA are isomers.

Third, biological human samples, such as CSF and plasma, on a practical level, undergo at least one freezing and thawing cycle before being analyzed. In addition, routines in the clinic may also lead to samples standing on the bench at room temperature before being frozen. Such handling may affect the concentrations of kynurenines. Knowing the stability of kynurenine pathway metabolites, in order to avoid those variances, is important when reporting high quality data both in the clinic and in experimental research. However, the stability of kynurenine pathway metabolites has previously been studied. KYNA has been shown to be stable in human urine up to 36 h when stored at 4°C (Furlanetto et al., 1998). In acidified

authentic human urine, tryptophan, kynurenine, KYNA, and AA have been shown to be stable for up to 3 cycles of freeze-thaw and up to 90 days at -20° (Yan et al., 2017). AA in human blood is stable for only 30 min at room temperature, while tryptophan, kynurenine, KYNA, 3-HK, and XA are stable for up to 4 hours (Boulet et al., 2017).

To date, tryptophan and kynurenine have mainly been analyzed using high performance liquid chromatography (HPLC) with electrochemical detection, ultraperformance liquid chromatography (UPLC), gas chromatography-mass spectrometry (GS-MS), and liquid chromatography-mass spectrometry (LC-MS) (Linderholm et al., 2012; Notarangelo et al., 2012; Jones et al., 2015; Flieger et al., 2018). KYNA has mainly been analyzed using HPLC (Shibata, 1988; Swartz et al., 1990; Jauch et al., 1993; Erhardt et al., 2001) with fluorescence detection and QUIN and PIC using GC-MS (Smythe et al., 2002; Erhardt et al., 2013; Brundin et al., 2016).

Thus, a sensitive, robust, and specific method for simultaneously detection and quantification of multiple kynurenine metabolites in human CSF and plasma has been lacking.

Given that kynurenine pathway metabolites are suggested to play a role in the pathophysiology of psychiatric disorders it is of high interest to investigate their relation between CSF and plasma. Since the CSF is not easily accessible, it is a clinical advantage for psychiatric disorders to have blood biomarkers. In addition, since kynurenines might have the potential as future biomarkers used in clinical situations, there is a need to investigate how they are affected by confounding factors. Importantly, many studies show that physical exercise affects kynurenines in the periphery (Lim et al., 2021). Currently, there is a need for understanding how physical exercise affects kynurenine pathway metabolites centrally, and to what extent this is reflected in the periphery.

1.3 Psychiatric disorders and the kynurenine pathway

1.3.1 Bipolar disorder

Bipolar disorder is a severe chronic psychiatric disease. Based on data from the World Health Organization (WHO) up to 60 million people suffer from the disorder that is characterized by mood disturbances, with recurring episodes of either *mania* (elevated mood, reduced need for sleep, increased energy) or *hypomania* (milder symptoms than mania) and episodes of *depression* (low mood, decreased energy, loss of pleasure) (Phillips & Kupfer, 2013). Between

the depressive and manic episodes, the majority of patients experience a neutral mood, referred to as the euthymic state. According to the latest version of the main diagnostic manual for psychiatric disorders, Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-V) there are four main subtypes of bipolar disorders (American Psychiatric Association, 2013):

1. Bipolar disorder type I – at least one episode of mania, that might have been preceded or followed by an episode of hypomania or an episode of major depression.
2. Bipolar disorder type II – at least one episode of hypomania and one major depressive episode (and no episodes of mania).
3. Cyclothymic disorder – hypomanic symptoms and depressive symptoms, but not full episodes of hypomania and episodes of major depression.
4. Bipolar disorder not otherwise specified – hypomanic and depressive–like episodes, that might change rapidly, but do not correspond to bipolar disorder type I, to bipolar disorder type II or cyclothymic disorder.

The diagnosis of bipolar disorder is based on symptomatology and clinical phenomenology, rather than on underlying biological mechanisms. To date, unfortunately, there are no biomarkers developed, neither for predicting and diagnosing the disorder nor for evaluating the treatment response. The lifetime prevalence of bipolar disorder is estimated to be 2.4% and the one-year prevalence is estimated to be 1.5% (Merikangas et al., 2011). The disease is associated with increased morbidity and mortality (Merikangas et al., 2011). Indeed, patients with bipolar disorder have a lifespan reduction compared to the general population (Crump et al., 2013). Suicide occurs often among patients with bipolar disorder. Around 30-50% of patients with bipolar disorder attempt suicide and 15-20 % of them die by suicide (Dong et al., 2019). Importantly, bipolar disorder has the highest rate of suicide among all psychiatric diseases (Miller & Black, 2020).

Pathophysiology

What exactly causes the bipolar disorder is not known (Haggarty et al., 2021). However, several risk factors, such as genetics, environmental factors, substance abuse, and stressful life events, have been suggested (Craddock & Sklar, 2013; Uher, 2014). Bipolar disorder has a strong genetic component. The number of suggested risk genes is growing, especially in unbiased studies, such as Genome-wide association studies (GWAS). Some of the single nucleotide polymorphisms (SNPs) identified for bipolar disorder are reported to be in the genes involved

in calcium signaling (e.g., CACNA1C) (Ferreira et al., 2008; Green et al., 2013; Ruderfer et al., 2014), secretory pathways (LMAN2L) (Charney et al., 2017), cell cycle control (MAD1L1) (Hou et al., 2016), cytoskeleton (ANK3) (Ferreira et al., 2008; Charney et al., 2017), transcription factor activity (NFIX) (Ikeda et al., 2018) and neuronal connectivity (ODZ4) (Sklar et al., 2011; Green et al., 2013).

Also, neurotransmitter imbalance in the brain can lead to bipolar disorder. Thus, dysregulations of monoamine neurotransmitters such as dopamine, serotonin, norepinephrine, and other neurotransmitters such as GABA and glutamate are suggested to be involved in the pathophysiology of bipolar disorder. The imbalance of monoamines in the central nervous system (CNS) is believed to relate to both manic and the depressive episodes. This hypothesis is supported by the mechanism of action of antidepressant medications (Fišar, 2013; Hillhouse & Porter, 2015), that act by increasing synaptic availability of serotonin and norepinephrine. Furthermore, dopaminergic system dysregulations might also be part of bipolar disorder (Brugue & Vieta, 2007; Gershon et al., 2007). A serotonin and norepinephrine deficiency plays a role in depression and mood and this has been proposed since the 1960's (Bunney & Davis, 1965; Schildkraut, 1965). Furthermore, the balance of excitatory and inhibitory neurotransmitters, glutamate and GABA, is suggested to be of importance in the pathophysiology of bipolar disorder (Lan et al., 2009). Aberrant glutamate signaling in patients with bipolar disorder has been reported. Thus, a meta-analysis has revealed higher levels of glutamine and glutamate, measured by magnetic resonance spectroscopy, in the brain of patients with bipolar (Gigante et al., 2012). In addition, increased glutamate is also found in the post-mortem brain of bipolar patients (Lan et al., 2009). One study in patients with bipolar disorder, where both peripheral and central glutamate was measured, showed no differences when compared to healthy controls, whereas concentrations of glutamine, glycine, and D-serine were increased and L-serine decreased in the periphery; no changes were seen centrally (Pålsson et al., 2015). Increased glutamate concentration has been also found in the plasma of patients with a mood disorder (Mauri et al., 1998).

Brain immune activation is suggested to participate in the pathophysiology of bipolar disorder. According to studies revealed by St. Göran Bipolar Project, the pro-inflammatory cytokine, IL-1β is found elevated in the CSF (Söderlund et al., 2011) of these patients. Recently, it has been reported that variations in the pro-inflammatory IL-1β gene examined concerning different brain region volumes did not differ between healthy controls and patients with bipolar disorder of the same cohort (Strenn et al., 2021). In this thesis, bipolar disorder patients are the same subjects, but the data reported come from the 7-year follow-up.

Mounting evidence shows dysregulation of the kynurenine pathway in bipolar disorder. Indeed, elevated central levels of kynurenine and KYNA in subjects with bipolar disorder with a history of psychosis have been reported in several studies (Miller et al., 2004; Miller et al., 2006; Olsson et al., 2010; Lavebratt et al., 2014; Sellgren et al., 2016; Sellgren et al., 2019). Interestingly, CSF KYNA inversely correlates to depressive symptoms in patients with depression and suicidal behavior (Bay-Richter et al., 2015). An abnormal kynurenine pathway is also present in the periphery of patients with bipolar disorder (Bartoli et al., 2020) with decreased KYNA concentration being reported (Birner et al., 2017; Liu et al., 2018; Poletti et al., 2018; van den Ameele et al., 2020). Peripheral levels of QUIN in patients with bipolar disorder have been reported to be lower (Liu et al., 2018) or not changed when compared to healthy subjects (van den Ameele et al., 2020). To our knowledge, CSF QUIN has not been analyzed in bipolar disorder before. Plasma PIC levels are found to be lower in patients with bipolar disorder than in healthy subjects (Aarsland et al., 2019; Ryan et al., 2020).

Treatment

Pharmacological treatments for both bipolar disorder and psychiatric diseases, in general, have many limitations: the onset of treatment takes several weeks (Pjrek et al., 2009), many patients are resistant to the treatment, and the side effects reduce the patients' daily life quality. The most commonly used drugs for bipolar disorder are mood stabilizers. Antidepressant drugs are often prescribed to patients with bipolar disorder. To date, anti-epileptic medications, valproate, and lamotrigine as well as second generation antipsychotic drugs, risperidone, and olanzapine are also used in the treatment of the bipolar disorder. Lithium is the most common mood stabilizer used and also has anti-suicidal effects through an unknown mechanism (Schou, 2000). Lithium may act by stabilizing normal fluctuations in the transduction pathways of intracellular signals. Understanding the mechanism of action of lithium could lead to the identification of new drug targets for bipolar disorder. In the treatment of bipolar disorder, ebselen, has been shown to be a safer alternative to lithium (Singh et al., 2013). A recent study in rodents showed that acute administration of ebselen reduced levels of synaptic glutamate (Mota et al., 2020).

1.3.2 Suicide

In the Merriam-Webster Dictionary, the definition of suicide is "*the act or an instance of taking one's own life voluntarily and intentionally*". Suicide counts for approximately 1.5% of all deaths worldwide (Fazel & Runeson, 2020). According to WHO, around 800 000 people commit suicide every year, which means every 40 seconds a suicide occurs in the world. Moreover, according to the WHO data, suicide was the third leading cause of death among young adults aged 15-19 in 2019. In addition, males commit suicide more often than females, conversely females attempt suicide more often than males. However, the proportion of males and females vary depending on the method used, help-seeking, and culture differences (Canetto & Sakinofsky, 1998; Nordentoft & Branner, 2008; Mergl et al., 2015; Schaffer et al., 2015). Today, the most important and highest risk factor for suicide is a previous suicide attempt (Suominen et al., 2004; Runeson et al., 2010; Goñi-Sarriés et al., 2018). The lack of biomarkers for suicidal ideation, makes it challenging to prevent.

The majority of suicides are related to psychiatric disorders, like major depressive depression, bipolar disorder, and schizophrenia. Among all suicide cases, 3.4-14% suffer from bipolar disorder (Schaffer et al., 2015).

Importantly, according to a review published in 2019, around 80% of subjects that committed suicide contacted the primary health care one year prior to suicide, 40% contacted the primary health care the last month of their life, while 31% contact the mental health care professionals during the last year of their life (Stene-Larsen & Reneflot, 2019). Thus, improving risk assessments and developing tests, biological or psychological, can help health care professionals to identify high-risk subjects. The development of robust and reproducible biomarkers for suicidality will save lives.

Pathophysiology

The pathophysiology behind suicide behavior is very complex and multifactorial. To date the biology behind is far from being clear, however, neurobiological systems and genetics are constantly being explored. Several risk genes have been identified in suicidality. For instance, the malfunctioning of the serotoninergic system in suicidality is thought to be genetically controlled (Bondy et al., 2006). Strong gene candidates are the ones that code for proteins involved in the regulation of serotonin neurotransmission, such as tryptophan hydroxylase, serotonin transporter, serotonin receptor, and monoamine oxidase (MAO) A (Mann et al., 2001; Antypa et al., 2013).

Almost three decades ago, elevated soluble IL-2 receptors were found in suicide attempters. This study was indeed one of the first pieces of evidence suggesting an imbalanced immune system in suicide (Nässberger et al., 1993). Later, chronic administration of the inflammatory cytokine INF-α has been shown to induce suicidal ideation (Dieperink et al., 2004). Many studies have reported aberrant levels of immune markers centrally, as well as in the periphery. For instance, elevated CSF IL-6 (Lindqvist et al., 2009) has been found in suicide attempters. Moreover, IL-8 and vascular endothelial growth factor (VEGF) is decreased in the CSF of suicide attempters (Isung et al., 2012). In the periphery, increased plasma IL-6 and TNF-α levels have been found in suicide attempters (Janelidze et al., 2011), while IL-2 was found to be decreased in plasma of suicide attempters (Janelidze et al., 2011; Isung et al., 2012). The presence of a compromised immune system is confirmed also by postmortem studies. Thus, IL-6 mRNA levels are higher in the prefrontal cortex of suicidal subjects (Pandey et al., 2012) as well as in the hippocampus (Hoyo-Becerra et al., 2013). In the prefrontal cortex of suicidal subjects mRNA levels of IL-1β and TNF-α are also higher (Pandey et al., 2012), while increased mRNA levels of IL-4 and IL-13 have been seen in orbitofrontal cortex brain tissue of subjects that committed suicide (Tonelli et al., 2008).

Interestingly, in suicide attempters, a positive correlation has been found between CSF IL-6 and CSF QUIN (Erhardt et al., 2013). In the same subjects, CSF levels of QUIN are elevated in suicide attempters (Erhardt et al., 2013). Another closely related metabolite, PIC is found to be reduced in the CSF of the same patients (Brundin et al., 2016). While QUIN returns to normal levels 6 months after the suicide attempt (Bay-Richter et al., 2015), levels of PIC are constantly low up to two years after the suicide attempt (Brundin et al., 2016). In those subjects no changes in CSF KYNA are present, nevertheless the ratio QUIN/KYNA is increased in suicide attempters compared to healthy subjects (Erhardt et al., 2013). Another important ratio, kynurenine/tryptophan, representing the rate limiting enzyme activity of the pathway is elevated in suicide attempters (Brundin et al., 2016). In addition, the minor C allele of SNP rs2121337, located in ACMSD, the enzyme limiting QUIN generation by competitive production of PIC, was more prevalent in suicide attempters compared to healthy controls (Brundin et al., 2016). Thus, PIC might be used as a biomarker indicating vulnerability for suicide behavior, while a low ratio of PIC/QUIN or high QUIN might indicate acute risk of suicide.

Treatment

The lack of knowledge regarding pathophysiological mechanisms in suicidal behavior leads to limited options for pharmacological treatment. So far, ketamine, a glutamate signaling modulator involving NMDA and AMPA, is the most potent anti-suicidal treatment, its effects can be even be observed within hours. Several studies are being conducted in order to understand its mechanism of action. Studies from depressive-like behavior rodent genetic models (Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats) show that one single injection of ketamine leads to rapid *de novo* spine density growth and interestingly normalized increased levels of NMDA receptor subunits (Treccani et al., 2019). Other mechanisms have also been suggested from experimental research. For example, brain-derived neurotrophic factor (BDNF) has been suggested to be part of the mechanisms (Garcia et al., 2008; Autry et al., 2011). Activation of the mechanistic target of rapamycin (mTOR) pathways might also be one explanation of the ketamine mechanism of action (Liu et al., 2013; Zanos & Gould, 2018; Matveychuk et al., 2020).

1.4 Physical exercise impact on the kynurenine pathway

Accumulating evidence points to the beneficial effects of physical exercise on both health and disease. Interestingly, physical exercise has been shown to have a positive influence on the mental state of healthy subjects (Wollseiffen et al., 2016). Moreover, patients with the psychiatric disorder also show improvement in their symptoms following physical exercise. Specifically, physical exercise is a promising strategy to prevent the development of depressive disorders (Mammen & Faulkner, 2013; Schuch et al., 2018) and to improve depressive symptoms. Recently, the European Psychiatric Association suggested that physical activity should be part of standard prescriptions for treating depressive disorders (Wegner et al., 2014; Khanzada et al., 2015).

There is an increasing interest in the effects of physical exercise on the kynurenine pathway (Stubbs et al., 2018; Joisten et al., 2020). Indeed, an altered kynurenine pathway as reflected by increased or decreased kynurenine pathway metabolites has been suggested in several studies. These studies include different types of exercise. For example, aerobic exercise such as a marathon race (42.2 km) and 30 minutes of cycling or resistance exercise of 4 x 8-10 repetitions at 70% one-repetition maximum (1RM) show decreased levels of plasma tryptophan in healthy subjects following such interventions (Lewis et al., 2010; Mudry et al.,

2016; Joisten et al., 2020). A decrease in plasma tryptophan was also seen following incremental cycle ergometer exercise in both trained athletes (Strasser et al., 2016), and in patients with relapsing remitting multiple sclerosis (MS) (Bansi et al., 2018). Plasma tryptophan was also decreased in patients with type 2 diabetes when cycling for 30 minutes (Bansi et al., 2018) and in patients with gastroesophageal junction cancer receiving chemotherapy following 12 weeks and two weekly sessions of 30 - 34 minutes of cycling combined with resistance training (Herrstedt et al., 2019). To our knowledge, only one study has reported increased levels of plasma tryptophan following 60 minutes of continuous treadmill exercise in elderly subjects (Melancon et al., 2014). Other studies have reported no change in plasma tryptophan levels of experienced triathletes participating in a half-ironman triathlon (Areces et al., 2015). No change in tryptophan was seen when healthy subjects performed endurance exercise of 45 minutes cycling (Joisten et al., 2020) when patients with major depressive disorder (MDD) and patients with somatization syndrome (mental disorder associated by multiple and recurring clinically significant complaints about somatic symptoms (Rief & Hiller, 1999)) followed one week of a daily 30 minutes fitness and stretching training (Hennings et al., 2013), and elderly people following a program consisting of 16 weeks of trademill training, thrice weekly, 6 km/ h for 45 minutes (Melancon et al., 2014). In addition, patients with pancreatic cancer following 6 months of two times/week supervised moderate to high progressive resistance exercise or unsupervised home-based resistance exercise did not show any changes of plasma tryptophan over time (Pal et al., 2021).

Metabolites produced along the kynurenine pathway of tryptophan degradation, such as kynurenine, KYNA, and QUIN, have also been the focus of several studies investigating the effects of exercise.

Moreover, both increased and decreased levels of kynurenine in plasma have been reported. Incremental cycle ergometer exercise increased levels of plasma kynurenine in trained athletes (Strasser et al., 2016). Also, 6 months of twice/week unsupervised homebased ressitance exercise increased levels of plasma kynurenine over time in patients with pancreatic cancer (Pal et al., 2021). On the other hand, 30 minutes of cycling decreased the plasma kynurenine levels in healthy and type 2 diabetes subjects (Mudry et al., 2016). Plasma kynurenine levels show no change in secondary progressive and relapsing remitting MS patients after incremental cycle ergometer exercise (Bansi et al., 2018), in healthy subjects performing resistance exercise or endurance exercise (45 min cycling) (Joisten et al., 2020), in healthy subjects, patients with MDD and patients with somatization syndrome following one week of daily 30 minutes fitness and stretching training (Hennings et al., 2013), in elderly at risk of dementia after a 10 weeks

program of physical training (a mixed program of endurance, balance, coordination, strength exercises) (Küster et al., 2017), in patients with mild to moderate depression following 12 weeks of light, moderate or vigorous exercise (Millischer et al., 2017), in elderly subjects and in patients with gastroesophageal junction cancer receiving chemotherapy following 12 weeks of combined cycling and resistance exercise (Allison et al., 2019; Herrstedt et al., 2019).

Moreover, several studies have investigated the impact of physical exercise on KYNA levels in plasma. Different types of physical exercise, such as marathon race, half marathon race, 150 km road cycling, and 30 or 45 minutes cycling increase the levels of KYNA in plasma of healthy subjects (Lewis et al., 2010; Schlittler et al., 2016; Joisten et al., 2020), as well as 30 minutes cycling in type 2 diabetes subjects (Mudry et al., 2016). On the contrary, 100 drop jumps did not affect plasma KYNA levels in healthy subjects (Schlittler et al., 2016). No effect on KYNA was neither seen when elderly at risk of dementia performed 10 weeks program of physical training (a mixed program of endurance, balance, coordination, strength exercises) (Küster et al., 2017), or in mild to moderate depressed patients following 12 weeks of light, moderate or vigorous exercise (Millischer et al., 2017), or in elderly and patients with gastroesophageal junction cancer receiving chemotherapy following 12 weeks of combined cycling and resistance exercise (Allison et al., 2019; Herrstedt et al., 2019).

Some studies have also investigated how plasma levels of QUIN are affected by physical exercise and both increase and no change have been observed. In healthy subjects, levels of plasma QUIN were increased from a marathon race (42.2 km) (Lewis et al., 2010), 150 km of road cycling time trial (Schlittler et al., 2016), or 45 minutes of cycling (Joisten et al., 2020). In healthy subjects, plasma QUIN levels did not change following 100 drop jumps (Schlittler et al., 2016), or following resistance exercise (Joisten et al., 2020). No changes were neither seen in the elderly and elderly at risk of dementia following 12 weeks of combined cycling and resistance exercise, and 10 weeks program of physical training (a mixed program of endurance, balance, coordination, strength exercises) respectively (Küster et al., 2017; Allison et al., 2019).

The effects of physical exercise on neuropsychiatric disorders are of interest. Those effects seem to be linked to immune markers (Phillips & Fahimi, 2018; Ignácio et al., 2019), however, an exact mechanistic explanation of the relation between physical activity, immune markers, and clinical effects are missing. One study in rodents suggests a putative mechanism for this effect. Thus, exercise stimulates skeletal muscle expression of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α1) and increases the expression of the four KAT enzymes, that transform kynurenine into KYNA (Agudelo et al., 2014). This may

be attributed to lower levels of pro-inflammatory cytokines in the hippocampus of the same mice (Agudelo et al., 2014). Another study also reported increased gene and protein expression of KAT I, II, III, and IV enzymes in the muscles of subjects after endurance training, 150 km road cycling (Schlittler et al., 2016). One recent study showed that mice exposed to chronic mild stress had increased kynurenine and decreased KYNA in plasma (Fang et al., 2021). Interestingly, when those mice were treated with caffeine, gene expression of KAT I in skeletal muscles was upregulated, and PGC-1α1 was upregulated too. Thus, this study suggests that caffeine protects mice from stress-induced depression by upregulating PGC-1α1, which induces KAT 1 and hence restores kynurenine levels in the skeletal muscle (Fang et al., 2021). This might lead to a clearance of kynurenine from the plasma and thereby less kynurenine is transported through the BBB into the brain.

Moreover, supporting evidence of changes in immune markers has also been seen in healthy subjects (Gonçalves et al., 2020). Thus, pro-inflammatory cytokines, such as IL–12, IL–17, IL–18, TNF–α, INF–γ, chemokine monocyte chemotactic protein-1 (MCP–1), and anti-inflammatory cytokines such as IL–2R, IL–4, IL–5, IL–10, IL–13 were increased after a first soccer game in the plasma of female soccer players but were not affected after the second game separated by 72 hours recovery (Andersson et al., 2010). Nevertheless, the central modulation of immune markers following exercise is limited. One study reported minimal changes in concentrations of 17 of the 174 cytokines tested in CSF of healthy volunteers after an acute bout of intense aerobic exercise (Schön et al., 2019). Another study reported no changes of IL-6 in the CSF of healthy controls, while in plasma IL-6 was increased after cycle ergometer exercise (Steensberg et al., 2006).

Effects seen from exercise are a result of communication between body and brain (Cooper et al., 2018). However, in biomarker research, it's unclear how exercise affects immune markers and kynurenine pathway metabolites centrally, and to what extent this is reflected by changes in the periphery.

2 RESEARCH AIMS

Overall aim of the thesis

Develop a sensitive method for measuring kynurenine pathway metabolites in human CSF and plasma in order to investigate them as potential future biomarkers for psychiatric disorders and for understanding how physical exercise affects them.

Specific aims

1. Develop and validate a biochemical method for simultaneous quantification of kynurenine metabolites in human CSF and plasma that is highly sensitive, robust, and reliable.

2. Confirm previous observations of abnormal PIC/QUIN ratio in the CSF of suicide attempters compared to healthy controls in a small well-characterized cohort.

3. Analyze kynurenine metabolites in the CSF of patients with bipolar disorder.

4. Investigate if concentrations of kynurenine metabolites are linked to a history of suicidal behavior in patients with bipolar disorder.

5. Investigate possible associations between kynurenine metabolites in the CSF of patients with bipolar disorder with their pharmacological treatment and also with symptoms.

6. Investigate whether ACMSD gene variants affect PIC, QUIN, or the PIC/QUIN ratio in patients with bipolar disorder, without or with suicidal behavior.

7. Investigate how SIE affects kynurenine metabolites in plasma of healthy subjects of different age groups.

8. Investigate how aerobic exercise affects kynurenine metabolites and immune markers in CSF and plasma of healthy subjects with different exercise habits.

3 MATERIALS AND METHODS

3.1 Ethical considerations

Human studies are approved by Regional Ethical Review Boards in Lund, Sweden for Paper I (Dnr 150/2018); in Stockholm, Sweden for Paper II and IV (Dnr 2014/1201-31/1 and Dnr 2005/554-31/3) and in Kaunas, Lithuania for Paper III (BE 2-5).

All the studies were carried out following "The code of ethics of the world medical association" (Declaration of Helsinki) for experiments including human subjects. Every participant in the studies received verbal and written information about the research project and provided oral and written informed consent to participate in the study.

All CSF and plasma samples used in these studies are coded with unique barcodes. All personal data of the participants are coded, encrypted, and separately stored. Transfer of information about subjects between researchers is carried strictly following the requirements and conditions in The General Data Protection Regulation (GDPR).

3.2 Human subjects

3.2.1 Stability cohort

Ten patients with multiple sclerosis (MS) at the Karolinska University Hospital's MS outpatient clinic were asked to participate in the study between December 2017 and March 2019. Including criteria were: 1. no significant somatic or psychiatric diagnoses other than MS; 2. no psychiatric or psychotropic drugs; 3. being between the ages of 18 and 55 years old. Six subjects provided both CSF and blood samples and the other four subjects only CSF.

Four healthy volunteers enrolled in the Karolinska Schizophrenia Project (KaSP) in 2019 provided blood samples for the study.

3.2.2 Suicide cohort

Between 2009 and 2012, thirteen patients were admitted to Lund University Hospital after attempting suicide. A psychiatrist diagnosed all patients using the DSM IV soon after the suicide attempt. The doctor made his diagnosis a few days after the suicide attempt, after a long

structured interview that lasted about two hours using the Comprehensive Psychiatric Rating Scale (Åsberg & Schalling, 1979) and the Structured Clinical Interview for DSM Disorders (SCID I and II).

The inclusion criteria were that the subjects had performed a suicide attempt with the aim to end their life. A suicide attempt was defined as "*situations in which a person has performed an actually or seemingly life-threatening behavior with the of jeopardizing his/her life*" (Beck et al., 1972). Subjects had a diagnose and suicidality was a cross-diagnostic phenomenon (Diagnostic and Statistical Manual of Mental Disorders [DSM] V).

Before being part of the study, the medical history of the subjects was investigated. To identify any participants that might have infections, all subject´s temperature was measured and the blood for erythrocyte sedimentation rate, C-reactive protein, and white blood cell count was screened.

When subjects were enrolled in the study, eleven of the thirteen patients were on medical treatment, 8 subjects were using antidepressants, 6 subjects were taking painkillers, 6 subjects were taking sleep aids, 5 subjects were taking antipsychotics, 4 subjects were taking anxiolytics and 3 of them were taking mood stabilizers.

3.2.3 St. Göran Bipolar Project

The St. Göran Bipolar Project (SBP) is a prospective and longitudinal study of euthymic patients with bipolar disorder. Patients were enrolled from the bipolar unit at the Northern Stockholm Psychiatric Clinic, Stockholm, Sweden. All patients met the criteria of the DSM-IV for being diagnosed with Bipolar Disorder type I, II, cyclothymic disorder, or bipolar disorder not otherwise specified. Moreover, the Montgomery - Åsberg Depression Rating Scale (MADRS) (Montgomery & Asberg, 1979) and the Young Mania Rating Scale (YMRS) (Young et al., 1978) were used to assessing the extent of ongoing depressive and manic symptoms in patients.

3.2.4 Healthy subjects

Paper I

Thirteen healthy controls were randomly selected by the Swedish population registry to become part of the study. Afterward, they were contacted by phone. They were all somatically healthy, and they did not suffer from any previous or ongoing psychiatric condition or substance abuse. Two subjects were taking pain killers, one subject was taking oral contraceptives and two of them were taking nonprescription allergy medications.

Paper II

Eighty healthy controls were randomly selected by Statistics Sweden (SBC, http://www.scb.se) registry to become part of the study. Afterward, they were contacted by mail. Subjects with neurological conditions, dementia, pregnancy, untreated endocrine disorders, and chronic systemic autoimmune disorders, except persons with controlled asthma and allergies, as well as any current psychiatric disorder including a family history of schizophrenia or bipolar disorder in first-degree relatives personality disorder, drug or alcohol abuse (based on DUDIT, AUDIT and serum levels of carbohydrate- deficient transferrin) were not included in the study. Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) was used to for psychiatric disorders screening.

Paper III

Twenty recreationally active healthy volunteers, all males, participated in the study. Subjects were not following structured physical activity. Subjects were allocated into 2 subgroups, young and old.

Paper IV

Healthy subjects were recruited from student campuses by advertising. In order to be part of the study subjects should be not younger than 18 years old. Subjects that wanted to be part of the study were contacted by phone and afterward they were scheduled for an inclusion assessment with a consultant psychiatrist and their medical history was investigated. Subjects with psychiatric disorder, inflammatory, infectious, or autoimmune conditions could not be part of the study. MINI (Sheehan et al., 1998) was used to for psychiatric disorders screening.

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3.3 CSF and plasma

3.3.1 Lumbar puncture

Subjects in papers I, II, and IV went through a lumbar puncture. After a night of bed rest and fasting, a standardized CSF sampling occurred between 8.00 - 11.00 a.m. for subjects in paper I; 9.00 - 10.00 a.m. for subjects in paper II and 7.30 - 9.00 a.m. for subjects in paper IV. A non-cutting spinal needle was inserted between the vertebral interspaces L3/L4 or L4/L5 to collect CSF. To prevent gradient effects, the CSF sample was gently inverted and divided into 1.0 - 1.6 mL aliquots that were stored at -80°C until analysis (except for the samples that were used for stability testing).

3.3.2 Plasma isolation

Peripheral blood from subjects in papers III and IV was obtained. Blood collection was done using tubes containing ethylenediaminetetraacetic acid (EDTA) by using the standard venipuncture techniques. Blood was drawn in the morning between 7.30 and 9.00 a.m. for subjects in paper IV, after a full night of bed rest and fasting since midnight. Plasma was isolated by centrifugation at 2900 rpm for 15 minutes within 1 hour of blood collection and stored at -80°C pending analysis.

3.4 UPLC–MS/MS – kynurenine metabolites analysis in CSF and plasma

3.4.1 Chemicals and reagent

Tryptophan, L-kynurenine, KYNA, 3-HK, XA, 3-HANA, pyridine-2,3-dicarboxylic acid (QUIN), PIC, NA, and NAA used for preparing the normal standard mixture for UPLC-MS/MS analysis were all purchased from Sigma-Aldrich (MO, USA).

Tryptophan-d_3, L-kynurenine-d_4, QUIN-d_3, [$^{13}C_6$], NAA, [$^{13}C_6$]NA, XA-d_4, 3-HK-d_3, and 3HANA-d_3 used for preparing the internal standard (IS) mixture were purchased from Toronto Research Chemicals, Canada (Toronto, Canada). Two IS, KYNA-d_5 and PIC-d_4 were purchased from C/D/N Isotopes Inc. (Quebec, Canada).

Methanol and formic acid 99% used for plasma preparations were LC-MS grade and they were purchased from Chromasolve, Honeywell, VWR International AB, (Stockholm, Sweden). Ammonia (32%) and $ZnSO_4$ used also for plasma preparations, were purchased from VWR and Sigma-Aldrich (MO, USA) respectively.

3.4.2 Standard solutions

Stock solutions of kynurenine, KYNA, 3-HK, XA, 3-HANA, QUIN, PIC, NA, and NAA at 100 µM and tryptophan in 1000 µM in water UPLC grade were prepared and then stored at -20°C. Afterward, a mixture of all stock solutions was prepared, generating a final solution of 100 µM for tryptophan and 10 µM for the other compounds. The second batch prepared had a final solution of 8.3 µM for nine of the compounds and ten times higher for tryptophan, 83 µM. The standard mix was aliquoted in volumes of 200 µL and stored in -80°C. Before each experiment, one aliquot of the standard mix was thawed and serial dilutions in water UPLC grade were prepared.

Stock solutions of l-kynurenine-d_4, KYNA-d_5, QUIN-d_3, [$^{13}C_6$], PIC-d_4, NAA, [$^{13}C_6$]NA, XA-d_4, 3-HK-d_3 and 3HANA-d_3 at 50 µM and tryptophan in 500 µM in water UPLC grade were prepared and then stored at -20°C. Afterward, a mixture of all stock solutions was prepared, generating a final solution of 5 µM with an exception of tryptophan-d_3, which had the final concentration of 50 µM. The second batch of IS had a final concentration of 4.1 µM for nine of the compounds and 41 µM tryptophan-d_3. The IS mix was aliquoted in volumes of 350 µL and stored in -80°C.

3.4.3 Analysis

The UPLC–MS/MS system used a Xevo TQ–XS triple quadrupole mass spectrometer (Waters, Manchester, UK) with a Z-spray electrospray interface and Waters Acquity UPLC I-Class FTN system (Waters, MA, USA). Data processing and acquisition was performed using the software package MassLynx v 4.1 SCN943 SCN979 (© 2016 Waters Inc.). The UPLC conditions were as follow: separation performed on an Acquity UPLC® HSS T3 column (1.8 µm, 2.1×150 mm) from (Waters, part number: 186003540) column temperature 50°C; guard column (Waters, Vanguard HSS T3 1.8 µm, 2.1×50 mm column, part number: 186003976) was installed to retain contaminants from the mobile phase. The mobile phase consisted as follow: (A) 0.6%

formic acid in water (UPLC grade) and (B) was 0.6% formic acid in methanol (UPLC grade). The flow rate was 0.3 mL/min and the run time for each sample was 13.0 minutes. The MS was operated in electrospray-positive multiple reaction monitoring (MRM) mode. The capillary voltage was set to +3.0 kV. The source temperature was 150°C, the cone gas flow was 150 L/h and the desolvation gas flow rate was 1000 L/H. The desolvation temperature was 650°C and detector gain 1 was used. The m/z for the MRM transitions of each individual analyte, along with optimal cone voltages and collision energies were determined by manual tuning using the instrument's built-in fluidics system (MassLynx v 4.1 software) (Table 1). A 10 µL/min flow of 100 ng/mL tuning solution was introduced to the mass spectrometer. This was combined with an LC flow of 0.2 mL/min and a composition of 20/80 mobile phase A / mobile phase B. The MRM transition providing the highest sensitivity was chosen as quantification trace for all compounds, except for tryptophan (and kynurenine too in case of plasma) where the second most intense transition was chosen. ^{13}C isotopes were selected to reduce overall signal intensity. This provided better linearity of the response over the calibration range, since the concentration of tryptophan in CSF samples (as well as kynurenine in plasma samples) are significantly higher than for the other analytes in this method. The dwell times for the MRM channels were automatically calculated by the software, giving a desired number of 15–20 data points across the chromatographic peak. Table 1 shows a summary of the precursor/product transitions for all compounds and their respective IS. The autosampler temperature was set at 5°C.

Compound / internal standards	Precursor ion mass	Product ion mass	Cone voltage (V)	Collision energy (eV)
Tryptophan	206.1	118	20	24
		146**	20	16
Kynurenine	209.1	94	20	12
		146	20	18
KYNA	190.1	116	30	28
		144	30	17
QUIN	168.1	78	20	18
		124	20	10
PIC	123.9	78*	30	16
		96	30	16
NAA	123	78	25	16
		80*	25	16
3HK	225.2	110.1	14	16
		162.1	14	16
NA	123.9	80*	25	16
		96	25	16
XA	206.1	160.0	25	16
		132.0	25	30
3HANA	154	108*	20	18
		136	20	10
Tryptophan-d_3	208.1	118.8	40	26
Kynurenine-d_4	213.2	94	30	15

KYNA-d_5	195	121	28	26
QUIN-d_3	171	81	20	18
PIC-d_4	128	82	4	17
NAA [$^{13}C_6$]	129.1	101	20	16
3HK-d_3	228.2	163	14	16
NIC [$^{13}C_6$]	130.1	85.2	32	20
XA-d_4	210.1	192	25	10
3HANA-d_3	157	83	24	24

Table 1. Transitions and mass spectrometry parameters for all the compounds and their internal standards.

*quantifying ion; ** MS transition using ^{13}C isotopes and product ion with low response were selected to reduce the overall signal intensity.

3.4.4 Methods validation

The methods were validated for calibration curve linearity, lower limit of quantification (LLOQ), selectivity, specificity, accuracy, precision, matrix effects and stability following the guidelines of bioanalytical method validation from European Medicines Agency (EMA) and the US Department of Health and Human Services Food and Drug Administration (FDA). The calibration curve linearity was tested using a standard mix of all metabolites diluted in water. The concentrations for CSF analysis ranged from 0.1 to 250 nM (for tryptophan concentrations ranged from 0.1 to 25 µM) and for plasma analysis concentrations ranged from 0.006 and 8.3 µM (for tryptophan the concentrations ranged from 0.06 to 83 µM). All the standard mix concentrations, human CSF and plasma sample were analyzed in duplicates. The standard curve was calculated by 1/X-weighted least squares linear regression of standard curve calibrator concentrations and the peak area ratios of analyte to IS. In order to estimate the limit of detection (LOD) and the LLOQ, a signal-to-noise (S/N) ratio of 3 and S/N ratio of 10, respectively, was used. The guidelines recommend accuracy and precision variations are ±15% (LLOQ: ±20%) of nominal concentrations for chromatographic assays. In this thesis, the selectivity for all metabolites was investigated by comparing chromatograms of CSF or plasma obtained from six different human samples spiked with a mixture of ten metabolites and IS to

make sure that it was free of interference at the retention time of the compounds. Spiked CSF and plasma in two different concentrations each were used in order to test the accuracy and precision of the assay (see Table 2). The assay accuracy is presented as percentage recovery and is calculated as follows: (100 × [measured C_{spiked} - $C_{nonspiked}$ / C_{spiked}]). The assay precision is expressed as percent relative standard deviation and calculated as follows: STDEV [Data Range]/AVERAGE [Data Range] *100). Intra-assay accuracy and precision results come from six analyses of the same sample within the same experiment. Inter-assay accuracy and precision results come from three different experiments running over 2 days. The matrix effect was calculated from the area of the IS in pooled CSF or plasma human sample (n = 6) in relation to the peak area of a prepared sample of pure water (n = 6), prepared exactly as the human sample, as (Area [sample] / Area [water] - 1) *100%. The matrix effect should be reproducible and consistent.

Compound	**Concentrations used to spike**			
	CSF		**Plasma**	
	Low (A)	**High (B)**	**Low (A)**	**High (B)**
Tryptophan	1.5 µM	15 µM	9 µM	40 µM
Kynurenine	50 nM	500 nM	1.5 µM	3 µM
KYNA	2 nM	20 nM	50 nM	500 nM
3-HK	5 nM	50 nM	50 nM	500 nM
XA	2 nM	20 nM	15 nM	150 nM
3-HANA	2 nM	20 nM	40 nM	400 nM
QUIN	20 nM	200 nM	300 nM	3 µM
PIC	20 nM	200 nM	70 nM	700 nM
NA	2 nM	20 nM	2 nM	20 nM
NAA	10 nM	100 nM	150 nM	1.5 µM

Table 2. Concentrations used to spike the CSF and plasma in order to investigate the accuracy and precision of the intra-assay and inter-assay.

3.4.5 Sample preparation

CSF

30 µL of human CSF sample, standard mix, or Quality Control sample was mixed with 30 µL of IS working solution for 15 seconds. The IS working solution was prepared by diluting of IS mix in a ratio of 1:5 with 5% formic acid in water. The mixing was done in LC-MS Certified Clear Glass 12 × 32 mm vials (Waters, PN: 186005662CV). Samples were transferred to the autosampler (5°C) and a volume of 3 µL was injected into the UPLC-MS/MS system.

Plasma

30 µL of human plasma sample, standard mix, or Quality Control sample was mixed with 30 µL of IS 0.5 µM in 10% ammonia for 15 seconds. In addition, 60 µL of 200 nM ZnSO4 (5°C) was added and then mixed for 15 seconds. This was followed by adding 30 µL of methanol (5°C) and mixed for 15 seconds. Afterward, the mixture was centrifuged at 2841×g for 10 minutes at room temperature. 30 µL of the supernatant obtained after the centrifugation was mixed with 30 µL of formic acid 5% in LC-MS Certified Clear Glass 12×32 mm vials (Waters, product no. 186005662CV). Samples were transferred to an autosampler (5°C) and a volume of 1.5 µL was injected into the UPLC-MS/MS system

3.4.6 Stability test

Kynurenine metabolites, both in CSF and plasma human samples, were investigated how stable they are when they have been held at room temperature for varying amounts of time and when undergoing multiple freeze-thaw. The storage times at room temperature and the number of freeze-thaw cycles were chosen to cover normal sample preparation, analysis, and storage conditions.

Precisely, CSF and plasma kynurenine metabolites stability before and after the first freezing was tested by analyzing samples directly after lumbar puncture and blood drawing (followed by plasma isolation), after 24 h at room temperature (before any freezing), and then again after one freeze cycle. Further, were tested the stability of kynurenines for 4 freeze-thaw cycles stored at -80°C. Moreover, CSF and plasma kynurenine metabolites stability were also tested for 30 minutes, 1, 2, 3, and 4 hours stored at room temperature.

The stability of the metabolites at a specific time or freeze-thaw cycle is expressed as the average percentage of the six or four individual CSF / plasma ± standard deviation (SD) in relation to the baseline mean concentration of the correspondent metabolite.

3.5 HPLC – analysis of neurotransmitters

3.5.1 Chemicals and reagents

The standard γ-aminobutyric acid (GABA) and amino acid mix solution containing glutamate and serine were purchased from Sigma-Aldrich (MO, USA).

Sodium acetate, tetrahydrofuran, and methanol 99% LC-MS grade used for preparing the mobile phases were purchased from Sigma-Aldrich (MO, USA) and Chromasolve, Honeywell, VWR International AB, (Stockholm, Sweden) respectively.

O-phthaldialdehyde, 2-mercaptoethanol, boric acid, NaOH, ethanol 99.5%, used for CSF preparations to analyze GABA, glutamate, and serine were purchased from Sigma-Aldrich (MO, USA).

3.5.2 Standard solutions

Stock solution for GABA was prepared at 100 μM in HCl 0.1M, glutamate and serine were prepared at 12.5 μM. All compounds were mixed generating a final solution of 10 μM for GABA and 20 μM for glutamate and serine in MilliQ water.

3.5.3 Analysis

HPLC was used for analyses of glutamate, GABA, and serine. HPLC was equipped with a gradient pump (Spectra System P4000, Waltham, MA, USA),), a degasser (Spectra System SCM 400), a Luna 100 C18(2) column (50 × 2 mm i.d., 5 μm particle size, Phenomenex, Torrance, CA, USA) and fluorescence detection (Jasco FP-920, Japan). The excitation and emission wavelength of 495 and 344 nm were used for analyses of glutamate, GABA, and serine, respectively.

The flow rate was 0.5 mL/min and 20 μL of CSF sample was injected manually into the HPLC system. Two mobile phases used consisted of (I) 0.04 M sodium acetate buffer with a pH=6.95, with 2.5% (v/v) methanol and 2.5% (v/v) tetrahydrofuran (II) methanol. HPLC system started with 100% mobile phase (I), the gradient changed to 70% mobile phase (I) and 30% mobile phase (II) from time 0 to 11 minutes, then the gradient changed to 10% mobile phase (I) and 90% mobile phase (II) from 11 to 13 minutes, at the 14th minute the gradient changed to 100% mobile phase (I) and conditions did not change until the next injection. The retention times were glutamate 2.8 minutes, serine 5.4 minutes and GABA 11.9 minutes. Signals from the detector were sent to the computer, where they were analyzed using the software Datalys Azur Software version 4.6.0.0 (Grenoble, France).

3.5.4 CSF sample preparation

In order to analyze glutamate, GABA, and serine, CSF human samples were mixed with O-phthaldialdehyde / 2-mercaptoethanol (OPA / 2-MCE) reagent for 60 seconds. The reagent was prepared by solving 27 mg OPA in 0.5 mL ethanol (99.5%) and then mixing it with 4.5 mL borate buffer (0.4 M boric acid adjusted pH 10.4 with NaOH) and 20 μL 2-MCE.

3.6 Proximity extension assay - immune markers analysis

Proseek® Multiplex Inflammation I$^{96\times96}$ proximity extension assay (PEA) was used to determine immune markers in CSF and plasma. PEA uses a proximity extension technology to enable a high throughput multiplex proteomic immunoassay (Olink, Uppsala, Sweden) (Assarsson et al., 2014). The panel consisted of cytokines, chemokines, and a selection of other immune-related proteins. In total 92 immune markers. The assay uses epitope-specific binding and hybridization of a set of paired antibodies linked to oligonucleotide probes, which can then be amplified using a quantitative polymerase chain reaction to quantify relative protein concentrations in terms of log base-two normalized protein expression (NPX) values.

3.7 Genotyping

Tag single nucleotide polymorphisms (SNPs) for ACMSD (± 20kB) were chosen using Tagger (HapMap analysis panel: CEU, r^2 threshold = 0.8). SNP genotyping (rs10176573, rs10496731,

rs10928521, rs11897287, rs1519304, rs16830596, rs16831005, rs16831432, rs1954874, rs2121337, rs4953936, rs6430484, rs6430538, rs6712534, rs6714498, rs6722883, rs7604436) were performed using 5´nuclease assay chemistry with custom competitive allele specific PCR (KASP) technology probes (He et al., 2014) at the Genomics Core Facility at the University of Gothenburg, Sweden.

3.8 Exercise protocols

3.8.1 Sprint interval exercise

The exercise bout consisted of 1 sprint interval exercise (SIE) session. The SIE began with an 8 minutes warm-up session cycling at a power (W) equal to the individual's body mass (kg). This is followed by 30-seconds all-out cycling bout at 0.7 Nm/kg body weight repeated 6 times and 4-minutes passive recovery periods in between (Place et al., 2015). Five mL blood sample was collected before SIE (baseline), 1 hour, and 24 hours after SIE. Study participants were not allowed to eat for at least 2 hours before the SIE.

3.8.2 Aerobic training

Study participants were divided into 2 sub-groups based on their physical exercise habits. To evaluate the exercise habits weekly frequency and intensity of exercise of the last 6 months was considered. The intensity of the exercise was evaluated using The Borg Rating of Perceived Exertion (RPE scale), which ranges from 6 (nothing at all) to 20 (maximum) (Borg, 1982). RPE score of 12–13 represent moderate intensity, RPE score of 14–17 represent vigorous and RPE score ≥18 represent near-maximal to maximal intensity (Garber et al., 2011). Study participants that for at least 3 times a week run for up to 60 min, performed high-intensity interval training or resistance training, were allocated to the acute exercise group. Acute exercise 4 consecutive days of running. Study participants allocated in the acute group had to run 4 consecutive days. The first and the third day they were instructed to do a 30-minute high-intensity interval-training i.e. 10 intervals of one-minute high intensity run (Borg RPE 18–19) followed by two minutes of low intensity run (Borg RPE (9–11). On second and fourth day, study participants were instructed to run for at least 60 minutes at a vigorous pace (RPE average 14–16). Study participants that exercised at least twice a week, taking power walking or slow jogging were allocated to the training exercise group. This included a minimum of 30 min

running at a vigorous intensity (Borg RPE 14–16) three times weekly for 4 weeks, i.e. a total of 12 sessions.

Subjects that participated in the study were instructed to refrain from any physical exercise for seven days prior to baseline sampling, with the goal of limiting the acute effects of recent exercise on biomarker levels. Before starting the exercise intervention, baseline blood and CSF samples were taken, and follow-up exercise samples were taken the day after the last exercise session.

3.9 Data analysis

Statistical analyses were performed using:

- GraphPad Prism 8 and 9 for Mac (GraphPad, La Jolla, Ca, USA) – Paper I, II, III, IV
- Statistical Package for the Social Sciences 20.0 and 25 for Mac (SPSS, IL, USA) – Paper I and II respectively
- R-programming language, version R-3.2.3 (Vienna, Austria) – Paper IV

Paper I

The nonparametric two-tailed Mann–Whitney U-test was used to compare age, BMI and concentrations kynurenine metabolites, between healthy controls and suicide attempters. The nonparametrical one-tailed Mann–Whitney U-test was used when analysis was hypothesis driven, as in case of CSF levels of QUIN, PIC as well as the ratios kynurenine/tryptophan, PIC/QUIN. Data are expressed as mean ± SD. Statistical significance was considered when $P < 0.05$.

Paper II

The normality distribution was determined using D´Agostino & Pearson normality tests. Comparisons between healthy controls and patients with bipolar disorder were performed using Chi-square tests, Mann–Whitney U -tests, linear mixed or logistic regression model. *Post-hoc* Bonferroni correction followed multiple comparisons of genetic data analysis. All reported P-values, except for CSF PIC levels, are two-tailed. Data are expressed as Median ± Inter Quartile Range (IQR). Statistical significance was considered when $P < 0.05$.

Paper III

The nonparametric two-tailed Mann–Whitney U-test was used to compare differences between young and old metabolite levels at baseline (data presented as mean ± SEM). Kruskal-Wallis followed by *post-hoc* Dunn's test was used to analyze how SIE affected the metabolites and each time after SIE was compared with the baseline. Method validation data are presented as mean ± SD. Statistical significance was considered when $P < 0.05$.

Paper IV

The multi-variable linear regression models adjusting for sex and was used for data analysis. The paired Student t-test was used to compare the differences in kynurenine metabolites and immune markers between baseline and follow-up. A multi-variable linear regression model adjusting for sex and age and along with curated markers of sample handling, chemokine (C–C motif) ligand 19 (CCL19) (CSF), and axin-1 (plasma) was used to control for potential effects of pre-analytical variability relating to potential differences in sample handling. Correlations were performed using the false discovery rate (FDR) model with a significance cutoff positive false discovery rate $P_{FDR}<5$. After correcting for multiple testing, statistical significance was considered when $P < 5 \times 10^{-5}$.

4 RESULTS AND DISCUSSION

4.1 UPLC–MS/MS method validation (Paper I, III)

The UPLC-MS/MS method for analyzing the kynurenine metabolites in human CSF and plasma was validated following the guidelines for bioanalytical method validation from US FDA (https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf) and EMA (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf). The main objective for both guidelines is to ensure highly reliable data. The guidelines do not only highlight the importance of the method´s sensitivity, accuracy, and precision, but they also stress the importance of understanding the biological variability in different matrixes (plasma, serum, cerebrospinal fluid, etc.), as well as the stability of the analyte during different handling conditions. In the following sections, we present data from different test validations on all these parameters.

4.1.1 Calibration curve linearity

The calibration curve should be tested within the concentration range found in the unique samples for which the system is being established for. The calibration curves must be continuous and repeatable.

CSF

We used a calibrator mixture of all metabolites to test the linearity. In this mixture, the concentrations of tryptophan were from 0.1 to 25 µM and the concentrations of other metabolites were from 0.1 to 250 nM. Within the concentration ranges tested, they all showed good linearity (see Table 3).

Plasma

A calibrator mixture of all metabolites was used also to test the plasma method calibration curve linearity. In this case, the concentrations of tryptophan were from 0.06 to 83 µM and of nine other metabolites from 0.006 to 8.3 µM. Within the concentration ranges tested, all ten metabolites' standard curves have good linearity (see Table 4).

4.1.2 Lower limit of quantification

The lower limit of quantification (LLOQ) represents the lowest concentration of the metabolite that can be quantified in a reliable way, having precision and accuracy that is acceptable. The LLOQ defines the sensitivity of the method. The limit of detection (LOD) is the lowest concentration of the metabolite which is possible to be detected and distinguished from the background noise. A signal-to-noise (S/N) is a unitless parameter that determines the relationship between an analytical signal and the mean noise level for a given sample. An S/N ratio of 10 was used to estimate the LLOQ. Here we also present the LOD, which is estimated by S/N of 3.

The LLOQ values for all metabolites in CSF and plasma are presented in Table 3 and Table 4, respectively. Of note, 3-HANA, XA and NA could be detected in less than 15% of the CSF samples analyzed, and NA could be detected in less than 30% of plasma samples analyzed, therefore they were not included in further analysis.

4.1.3 Selectivity and specificity

During the method validation, it is important to ensure that the method has the appropriate selectivity. The metabolite measured should be the intended one, without any interference with other compounds such as degradation products formed during sample preparation, structurally similar metabolites, or administrated drugs. The specificity of the current method included chromatographically separation of NA from PIC. These two metabolites have the same chemical formula $C_6H_5NO_2$, but not the same chemical structure, they are isomers. The carboxyl group (-COOH) in PIC's chemical structure is at the second position (Figure 2e), while in NA it is at the third position (Figure 2f).

As suggested by both EMA and US FDA guidelines, selectivity was tested using CSF and plasma from at least six individual subjects. These subjects represented both healthy subjects and patients with MS.

We tested the selectivity for all ten metabolites in plasma and CSF by comparing chromatograms from blank CSF/plasma with CSF/plasma spiked with a mixture of 10 metabolites to make sure that no interference is present at the respective retention time of each metabolite. Chromatograms of the metabolites were found to be free from any interference in both CSF and plasma. Importantly, PIC and NA showed a clear chromatographic separation in CSF (see Figure 2 a-d) and in plasma (see Figure 3 a-d)

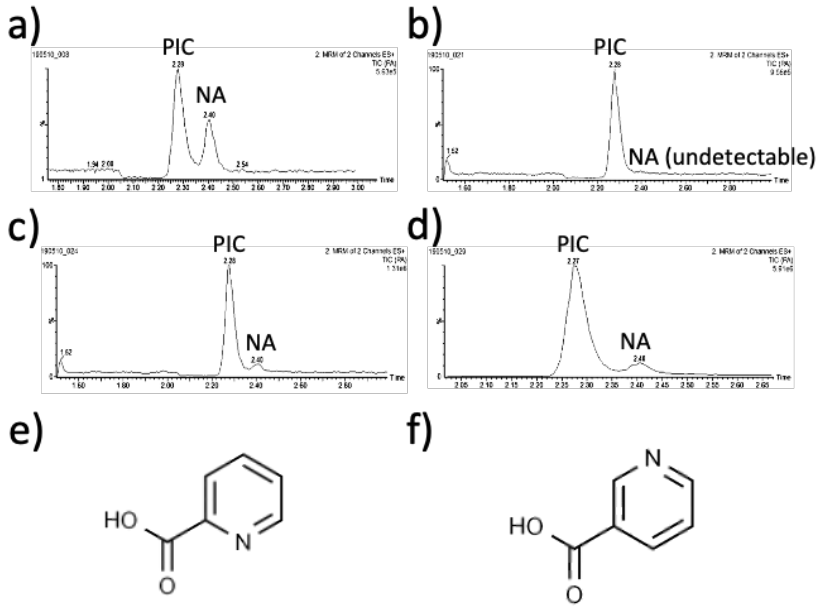

Figure 2. Chromatographically separation of PIC and NA in human CSF.

a) standard solution of 1 nM, b) CSF c) CSF spiked with 20 nM PIC and 2 nM NA, d) CSF spiked with 20 nM PIC and 20 nM NA, e) chemical structure of PIC, f) chemical structure of NA. ES+, TIC, MRM belong to PIC.

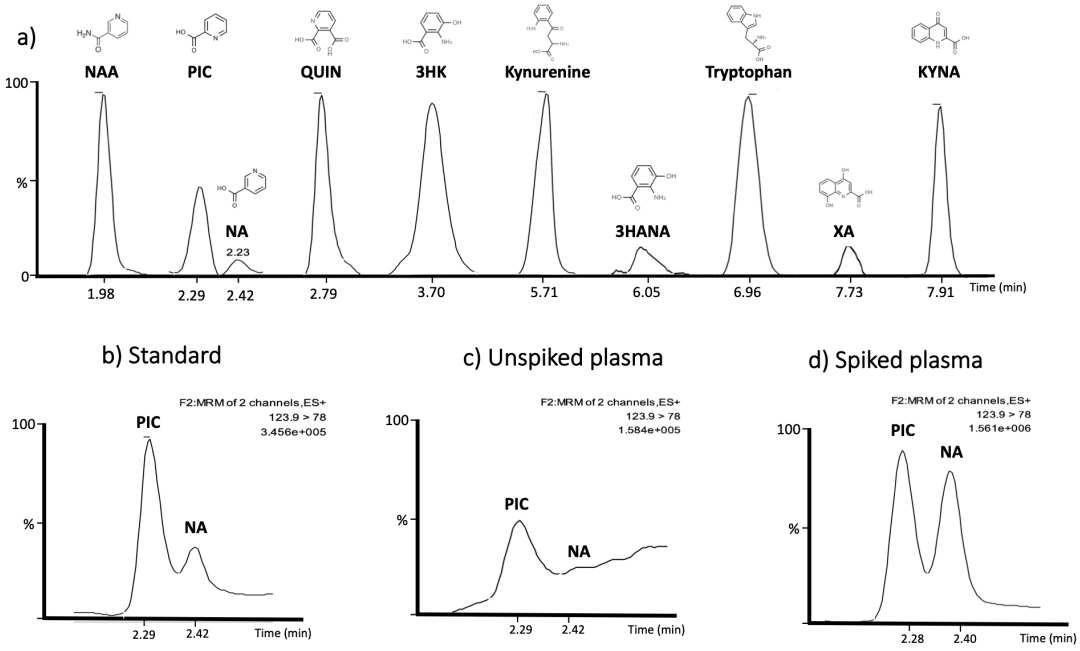

Figure 3. Chromatograms of kynurenines free from any interference and chromatographically separation of PIC and NA in human plasma.

a) Chromatograms of NAA, PIC, NA, QUIN, 3-HK, kynurenine, 3-HANA, tryptophan, XA, KYNA in human plasma, their chemical structures and their respective retention times; Chromatograms showing the separation of PIC from NA, b) in standard solution (6nM), c) in human plasma d) in spiked plasma showing that the separation is still present at higher concentration (1µM). ES+, TIC, MRM belong to PIC.

4.1.4 Accuracy and precision

The accuracy of the method presents the closeness of the determined concentration to the nominal concentration. The accuracy is present in percentage (%). The precision is the closeness of repeated measures of the metabolite. The precision is presented in relative standard deviation in percentage (RSD%). The method should be validated for inter-assay (between run) as well as intra-assay (within run) accuracy and precision. We tested the intra-assay accuracy and precision for six repeated measurements within one single assay and the inter-assay accuracy and precision for three different assays analyzed on two different days. We accepted variation less than 15% of the nominal value in line with the criteria of the US FDA and EMA guidelines.

CSF

Two different concentrations of spiked human CSF were used to assess accuracy and precision, and they were chosen to cover the range of concentrations found in human CSF. Nine of kynurenine metabolites showed satisfactory intra-assay accuracy with minor variations, ranging between 85% and 112%. All ten intra-assay precision values were satisfactory, between 1 and 9 RSD%. The inter-assay accuracy values were also in the acceptable range, between 86% and 113%. Furthermore, all inter-assay precision values were satisfactory, from 1 to 14 RSD%. The only metabolite with unsatisfactory variation in both intra- and inter-assay accuracy was 3-HANA, 134% and 140% of target tested, respectively, for the samples spiked with low concentration (see Table 3).

Plasma

The same procedure was followed also for plasma, and in this case, two different concentrations for spiking were chosen to cover the range of concentrations found in plasma. For all metabolites, the intra-assay accuracy and precision were satisfactory, ranging from 87% to 110% and 0.9 to 4.4 RSD%, respectively. The estimated inter-assay accuracy values for nine metabolites were between 85.2% and 114.8%, while the inter-assay precision values for all ten metabolites were between 1 and 14 RSD%. 3-HANA was the only metabolite that did not meet the inter-assay accuracy acceptance criteria (see Table 4).

4.1.5 Matrix effect

According to the EMA or US FDA bioanalytical method validation guidelines, the matrix effect is defined as "*a direct or indirect alteration or interference in response because of the presence of unintended analytes (for analysis) or other interfering substances in the sample*". The matrix effects on kynurenine metabolites in CSF and plasma were determined by calculating the ratio of the IS peak area mean of six CSF or plasma samples, to the IS peak area mean of the blank and were expressed in percentage. Matrix effects for CSF and plasma are presented in Table 3 and Table 4, respectively.

Compound	Linearity (R^2)	LOD-LLOQ	% Matrix effect	Intra-assay (n=6 during 20 h in +5°C)				Inter-assay (n=3)			
				Accuracy (% of target)		Precision (RSD%)		Accuracy (% of target)		Precision (RSD%)	
				Conc A	Conc B	Conc A	Conc B	Conc A	Conc B	Conc A	Conc B
Tryptophan	0.996	10–10 nM	-8.4	102	108	1	1	101	107	2	2
Kynurenine	0.997	0.1–0.25 nM	-4.1	96	115	1	1	95	114	1	3
KYNA	0.998	0.1–0.5 nM	-12.1	102	106	1	1	102	106	2	1
3-HK	0.997	0.25–1 nM	23.4	94	91	1	2	95	92	1	2
XA	0.989	2.5–10 nM	6.0	95	85	2	2	97	86	7	3
3-HANA	0.963	1–5 nM	-3.9	136	97	4	2	140	97	8	8
QUIN	0.998	2.5–5 nM	3.3	95	97	2	1	94	97	1	4
PIC	0.979	1–5 nM	12.5	112	89	3	5	113	87	4	1
NA	0.997	1–5 nM	-4.0	101	96	9	1	97	95	2	1
NAA	0.996	2.5–10 nM	2.4	94	93	3	1	92	92	14	3

Table 3. Linearity, LOD, LLOQ, matrix effect, accuracy and precision for intra-assay and for inter-assay results of validation of method for quantifying kynurenine metabolites in human CSF.

Concentration A and B can be found in Table 2.

Compound	Linearity (R^2)	LOD-LLOQ	% Matrix effect	Intra-assay (n = 6 during 20 h in +5°C)				Inter-assay (n = 3)			
				Accuracy (% of target)		Precision (RSD%)		Accuracy (% of target)		Precision (RSD%)	
				Conc A	Conc B	Conc A	Conc B	Conc A	Conc B	Conc A	Conc B
Tryptophan	0.999	6-6 nM	-38.6	108.2	100.4	1.2	0.9	102.6	85.6	6	6.4
Kynurenine	0.996	6-6 nM	-23.3	108.5	113.1	0.7	2.3	114.8	105.2	2.8	13.6
KYNA	0.999	6-6 nM	-36.7	90.7	91.8	1.8	0.6	87.9	98.0	10.8	6.9
3-HK	0.999	6-10 nM	-54.5	96.3	87.0	1.9	1.2	87.3	85.2	9.5	5.0
XA	0.999	6-10 nM	-18.5	100.6	100	5.2	1.4	86.3	105.6	3.7	6.5
3-HANA	0.999	6-10 nM	3.2	110.4	107.3	4.4	1.8	112.9	79.2	5.1	14.4
QUIN	0.999	6-10 nM	-30	87.3	93.9	2.7	2.6	114.7	103.2	1.9	10.6
PIC	0.995	10-10 nM	-50.5	90.2	99.8	2.4	0.9	86.9	96.0	6.5	7.6
NA	0.999	10-10 nM	-26.3	104.8	95.9	1.4	1.6	112.5	94.4	9.3	4.5
NAA	0.996	6-10 nM	-27.4	104.4	88.1	3.2	2.4	102.5	89.7	1.8	4.5

Table 4. Linearity, LOD, LLOQ, matrix effect, accuracy and precision for intra-assay and for inter-assay results of validation of method for quantifying kynurenine metabolites in human plasma.

Concentration A and B can be found in Table 2.

4.1.6 Stability

Stability testing should be performed to ensure that bench-top storage at room temperature during sample preparation, analysis, and the storage conditions used, do not affect the metabolite concentration. The stability of kynurenine metabolites in human CSF and human plasma was evaluated for bench-top storage at room temperature and repeated freeze-thaw cycles.

CSF

We used CSF from 6 individuals with MS to test the kynurenine metabolite´s stability. The mean baseline concentrations of kynurenine metabolites in the CSF of those six subjects are presented in Table 5. All metabolites were found to be stable when stored at room temperature for 30 minutes, 1, 2, 3, and 4 hours, showing an average variance of a maximum of 13% (see Table 5). An average variance of a maximum of 7% was also seen for all metabolites after 2-5 freeze-thaw cycles (see Table 6).

We used CSF from 4 additional individuals with MS to test kynurenine metabolites stability after the first freeze-thaw cycle. The mean baseline concentrations of kynurenine metabolites in the CSF of those four subjects are presented in Table 7. After one freeze-thaw cycle, the percent stability showing an average variance of a maximum of 7%. Storing samples at room temperature for 24 hours resulted in a reduction of 3-HK concentration by 25% but did not affect other metabolite levels.

Compound	Baseline Mean ± SD	Storage in room temperature (n=6) expressed in % change				
		30 min	1 h	2 h	3 h	4 h
Tryptophan	1.5±0.5 µM	105 ± 4.9	105 ± 4.9	99 ± 4.9	101 ± 4.9	105 ± 4.9
Kynurenine	60.2±30.6nM	95 ± 4.1	96 ± 4.1	93 ± 4.1	96 ± 4.0	100 ± 2.7
KYNA	2.4±0.8 nM	113 ± 4.9	102 ± 4.9	89 ± 4.9	100 ± 4.7	109 ± 4.7
3-HK	3.1±1.2 nM	99 ± 5.1	102 ± 2.9	97 ± 6.0	97 ± 4.1	102 ± 2.7
QUIN	23.6±19.7nM	103 ± 5.9	106.2 ± 1.6	100 ± 4.6	101 ± 4.4	108 ± 2.5
PIC	14.5±3.0 nM	98 ± 4.8	101 ± 4.2	96 ± 4.5	99 ± 4.0	104 ± 4.6
NAA	22.8±20.9nM	100 ± 6.1	102 ± 1.9	96 ± 6.4	103 ± 7.2	111 ± 7.2

Table 5. Percent stability of kynurenine metabolites in human CSF at room temperature after 30 min, 1 h, 2 h, 3 h and 4 h (mean ± SD).

Compound	Baseline Mean ± SD	Freeze-thaw (n=6) expressed in % change			
		Thaw 2	Thaw 3	Thaw 4	Thaw 5
Tryptophan	1.5 ± 0.5 µM	104 ± 4.9	100 ± 4.9	99 ± 4.9	101±4.9
Kynurenine	60.2 ± 30.6 nM	98 ± 4.1	94 ± 4.1	94 ± 4.07	95±4.1
KYNA	2.4 ± 0.8 nM	100 ± 4.9	96 ± 4.9	107 ± 4.9	101±4.9
3-HK	3.1 ± 1.2 nM	103 ± 2.9	97 ± 1.7	97 ± 1.7	96±1.7
QUIN	23.6 ± 19.7 nM	105 ± 1.5	101 ± 1.5	102 ± 5.9	100±5.9
PIC	14.5 ± 3.0 nM	101 ± 4.7	97 ± 4.7	99 ± 4.6	101±6.3
NAA	22.8 ± 20.9 nM	101 ± 2.3	98 ± 2.0	98 ± 7.6	105±6.6

Table 6. Percent Stability of kynurenine metabolites in human CSF after 2-5 freeze-thaw cycles (mean ± SD).

Compound	Baseline Mean ± SD	1st freeze-thaw cycle & storage in room temperature for 24 h (n=4) expressed in % change	
		Thawing 1	24 h in room temperature
Tryptophan	1.3 ± 0.1 µM	102 ± 3	95 ± 2.4
Kynurenine	67.7 ± 57.7 nM	104 ± 4	95 ± 8.8
KYNA	2.0 ± 0.4 nM	98 ± 2	99 ± 1.6
3-HK	4.4 ± 3.2 nM	93 ± 4	75 ± 4.0
QUIN	22.0 ± 3.6 nM	99 ± 2	111 ± 7.3
PIC	9.2 ± 4.2 nM	100 ± 3	92.7 ± 4.3
NAA	26.2 ± 10.4 nM	93 ± 8	104 ± 5.9

Table 7. Percent stability of kynurenine metabolites in human CSF directly after of lumbar puncture (30 min after), after 24 h at room temperature (before any freezing), then again after one freeze cycle (mean ± SD).

Plasma

The mean baseline plasma concentrations of the metabolites from six subjects with MS are presented in Table 8. The majority of metabolites (8 out of 10) were found to be stable at room temperature up to 4 hours, showing an average variance of a maximum of 4%. XA and 3-HANA were stable at room temperature for only up to 2 hours and showed an average variance of a maximum of 15% (see Table 8). An average variance of a maximum of 7% was also seen for all metabolites but XA, after 2-4 freeze-thaw cycles. Multiple freeze-thaw cycles reduced levels of XA by 20% (see Table 9).

We used plasma from four healthy subjects to investigate the stability before the first freeze-thaw cycle and after 24 h being stored at room temperature. Table 10 shows the mean baseline concentrations in fresh human plasma, analyzed within 30 minutes of blood drawing (followed by plasma isolation). The majority of metabolites (8 out of 10) were found to be stable when stored for 24 hours at room temperature showing an average variance of a maximum of 11%. QUIN and XA, on the other hand, increased by 32% and decreased by 30% respectively (see Table 10).

All metabolites were stable after the first freeze-thaw cycle, having an average variance of a maximum of 10%. However, 3-HANA was an exception showing 26% average variation (see Table 10). The percent rise in 3-HANA was found to be similar in all four subjects.

Compound	Baseline Mean ± SD	Storage in room temperature (n=6) expressed in % change				
		30 min	1 h	2 h	3 h	4 h
Tryptophan	34.9 ± 4 μM	102.2 ± 2.9	102.3 ± 2.9	99.5 ± 2.5	100.5 ± 1.6	101.5 ± 1.9
Kynurenine	2.4 ± 0.3 μM	102.4 ± 6.0	101.8 ± 4.8	99.6 ± 5.8	99.1 ± 1.7	101.1 ± 6.5
KYNA	34.6 ± 13.3 nM	97.3 ± 7.1	97.1 ± 5.9	96.9 ± 7.0	96.7 ± 6.8	98.1 ± 6.1
3-HK	27.8 ± 5.1 nM	100.7 ± 2.7	100 ± 4.6	97.5 ± 6.5	98.9 ± 3.2	102.3 ± 7.5
XA	14.3 ± 7.6 nM	95.1 ± 52.1	85.7 ± 40.1	88.9 ± 42.7	80.5 ± 38.2	96.4 ± 54.8
3-HANA	43.7 ± 37.5 nM	106 ± 10.3	104.7 ± 12.8	114.3 ± 12.7	109.7 ± 11.9	119.1 ± 13.2
QUIN	161.7 ± 37 nM	102.1 ± 5.1	103.1 ± 6.1	100 ± 3.8	100.8 ± 5.7	103.6 ± 6.1
PIC	36.8 ± 14.3 nM	100.2 ± 5.2	99.2 ± 3.4	100.9 ± 3.0	97.4 ± 5.6	99.5 ± 5.8
NAA	178 ± 76.8 nM	101.7 ± 3.8	101.5 ± 4.9	101.7 ± 3.4	100.5 ± 4.4	101.4 ± 5.1

Table 8. Percent stability of kynurenine metabolites in human plasma stored at room temperature after 30 min, 1 h, 2 h, 3 h and 4 h (mean ± SD).

Compound	Baseline Mean ± SD	Freeze-thaw (n=6) expressed in % change		
		Thaw 2	Thaw 3	Thaw 4
Tryptophan	34.9 ± 4 μM	100.5 ± 1.9	99.9 ± 4.5	101 ± 1.4
Kynurenine	2.4 ± 0.3 μM	101 ± 5.4	102.9 ± 6.5	100.1 ± 3.1
KYNA	34.6 ± 13.3 nM	99.3 ± 2.4	99.7 ± 5.8	95.3 ± 7.9
3-HK	27.8 ± 5.1 nM	101.3 ± 3	103.2 ± 3	97.8 ± 2.5
XA	14.3 ± 7.6 nM	90.8 ± 49.6	86.7 ± 39.9	80.8 ± 40.2
3-HANA	43.7 ± 37.5 nM	103 ± 6.8	106.7 ± 13.3	104.3 ± 9.5
QUIN	161.7 ± 37 nM	100.5 ± 3.4	103.1 ± 9.8	98.4 ± 4.2
PIC	36.8 ± 14.3 nM	100.1 ± 2.1	100 ± 3.5	101 ± 2
NAA	178 ± 76.8 nM	101.8 ± 3.4	99.6 ± 4.4	102.5 ± 5.9

Table 9. Percent stability of kynurenines in human plasma after 2-4 freeze-thaw cycles (mean ± SD).

Compound	Baseline Mean ± SD	1st freeze-thaw cycle & storage in room temperature for 24 h (n=4) expressed in % change	
		Thawing 1	24 h in room temperature
Tryptophan	30.9 ± 5.3 μM	102 ± 1.3	98.0 ± 4.7
Kynurenine	2.2 ± 0.3 μM	105.9 ± 6.0	93.7 ± 4.4
KYNA	44.3 ± 10 nM	101.6 ± 2.2	97.6 ± 5.5
3-HK	28.8 ± 7.6 nM	99.9 ± 3.2	88.7 ± 1
XA	20.9 ± 6 nM	99.5 ± 2.7	69.5 ± 28.9
3-HANA	29.6 ± 6.5 nM	126.4 ± 2.0	111.7 ± 3.9
QUIN	144.5 ± 18.8 nM	110.2 ± 9.2	132.4 ± 5.1
PIC	80.3 ± 33.1 nM	98.7 ± 1.6	95.2 ± 4.6
NAA	130.2 ± 91.8 nM	107.7 ± 11.7	96.0 ± 0.04

Table 10. Percent stability of kynurenines in human plasma directly after of blood drawing (followed by plasma isolation) (30 minutes after), after 24 hours being stored at room temperature (before any freezing), and also after the first freezing cycle (mean ± SD).

4.2 Kynurenine metabolites in the CSF of patients with bipolar disorder and suicide attempters (Paper I, II)

4.2.1 Decreased ratio PIC/QUIN in the CSF of suicide attempters

In the CSF of 13 healthy controls and 13 suicide attempters, we successfully detected tryptophan, kynurenine, NAA, KYNA, 3-HK, QUIN and PIC. No differences regarding age, sex and BMI were found between healthy controls and suicide attempters (see Table 11). Suicide attempters had a mean of total SUAS score of 43.8 ± 17.0.

	Healthy controls	Suicide attempters	P value [a]
Age (years) [b]	40.4 ± 14.6	39.2 ± 15.6	0.49
Sex (male/female)	5/8	7/6	
BMI [c]	24 (19 – 30)	26 (19 – 51) [d]	0.023

Table 11. Demographic characteristics of the study population and kynurenine metabolites levels

[a] Mann-Whitney test; [b] Mean ± SD; [c] median, IQR: Inter quartile range; [d] data missing for one subject

Concentrations of tryptophan, kynurenine, NAA, KYNA, QUIN, 3-HK, PIC, and ratio kynurenine/tryptophan did not differ between suicide attempters and healthy controls (see Figure 4). The CSF PIC/QUIN ratio of suicide attempters was found to be lower than the CSF PIC/QUIN ratio of healthy controls (P = 0.03). Concentrations of CSF QUIN were higher in suicide attempters (P = 0.19) and concentrations of CSF PIC were lower in suicide attempters (P = 0.11), however, none reached statistical significance. Previously, we have shown that QUIN is elevated, and PIC is reduced in the CSF of suicide attempters (Erhardt et al., 2013; Brundin et al., 2016). Thus, CSF PIC and QUIN levels between suicide attempters and healthy controls were as hypothesized. The current findings support our original paper, where we also found a lower CSF PIC/QUIN ratio in suicide attempters (Brundin et al., 2016). However, studies in larger cohorts of suicide attempters are required to determine whether PIC, QUIN, or the ratio PIC/QUIN can be used as biomarkers in the clinic.

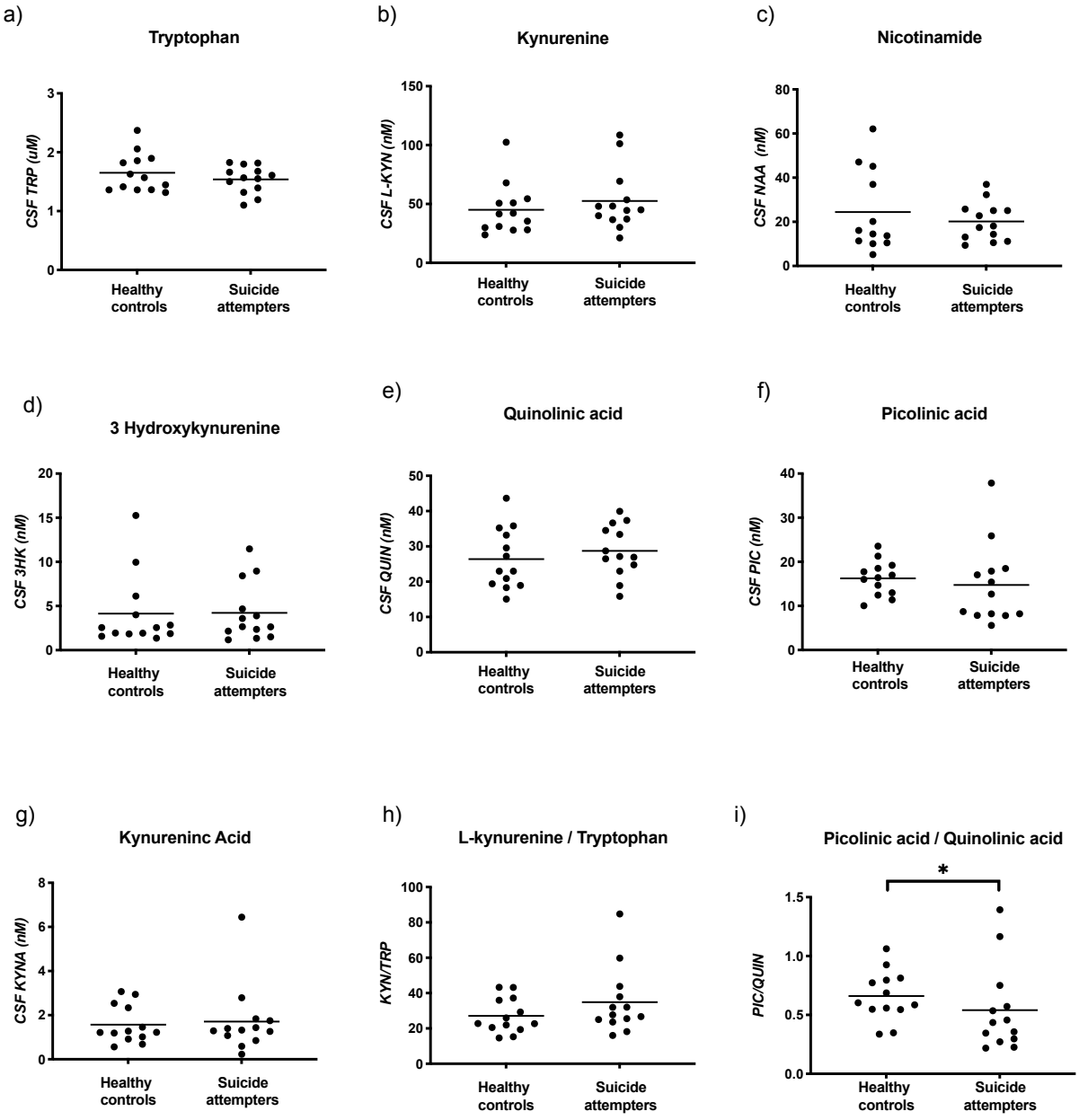

Figure 4. Cerebrospinal fluid levels of kynurenine metabolites in healthy controls and suicide attempters.

Levels of a) tryptophan, b) kynurenine, c) nicotinamide, d) 3-hydroxykynurenine, e) quinolinic acid, f) picolinic acid, g) kynurenic acid, h) ratio of kynurenine/tryptophan i) ratio of picolinic acid/quinolinic acid in the CSF of healthy controls and suicide attempters. Each point represents the concentration of a single CSF sample; the horizontal lines represent the mean for each group; Mann–Whitney test, *P < 0.05.

4.2.2 Kynurenine metabolites in the CSF of patients with bipolar disorder

We measured CSF levels of tryptophan, kynurenine, KYNA, QUIN, and PIC in the CSF of 101 patients with bipolar disorder and 80 healthy controls. Table 12 offers a summary of clinical and demographic characteristics of the study participants.

Characteristic	Median (IQR)		P-value
	Healthy Controls (n = 80)	Bipolar disorder patients (n=101)	
Age (years)	33 (27.3-43.8)	43 (35.5-54)	<0.0001 [a]
Sex (male/female)	39 / 41	40/61	0.22 [b]
BMI[1] (kg/m^2)	23.48 (21.6-25.7)	25.33 (22.4-38.8) [2]	0.003 [a]
Nicotine (yes/no)	17/ 63	39/59 [3]	0.008 [b]
Bipolar I disorder	-	n=45	-
Bipolar II disorder	-	n=43	-
Bipolar disorder NOS [4]	-	n=5	-
Other diagnosis		n=7	
Lithium	-	53%	-
Topiramate	-	2%	-
Clonazepam	-	2%	-
Lamotrigine	-	14%	-
Valproate	-	9%	-
Stimulant medication	-	17%	-
Antidepressant medication	-	42%	-
Anxiolytic medication	-	18%	-
Antipsychotic medication	-	33%	-
Sedative medication	-	39%	-

Suicidal behavior [5] (yes/no)	-	19/ 79 [3]	
MADRS [6]	0 (0-2) (80)	2.5 (0-5.75) (98) [3]	< 0.0001
YMRS [7]	0 (0-0) (80)	0 (0-1.75) (100) [2]	< 0.0001
CSF Tryptophan (μM)	1.5 (1.3-1.6) (80)	1.4 (1.2-1.7) (99) [8]	0.73 [c]
CSF L-kynurenine (nM)	32.9 (26.6-40.1) (80)	44.6 (32.5-62.1) (100) [2]	0.06 [c]
CSF KYNA (nM)	1.3 (0.9-1.7) (71) [9]	1.7 (1.2-2.4) (99) [8]	0.007 [c]
CSF PIC (nM)	10.2 (6.6-14.0) (78) [8]	13.3 (8.3-19.8) (101)	0.001 [c]
CSF QUIN (nM)	18.2 (13.4-23.2) (80)	21.2 (15.4-29.1) (100) [2]	0.48 [c]
CSF kynurenine / tryptophan	22.2 (18.6-26.8) (80)	31.86 (23.0-39.8) (99) [8]	0.04 [c]
CSF PIC / QUIN	0.5 (0.3-0.9) (78) [8]	0.5 (0.4-1.0) (100) [2]	0.02 [c]

Table 12. Clinical and demographic characteristics of the study participants

[a] Mann-Whitney test; [b] Chi-square test; [c] Linear Mixed Model [1] Body mass index; [2]Information missing for 1 subject; [3] Information missing for 3 subjects; [4] Bipolar disorder not otherwise specified; [5] Suicide attempt or self-harm; [6] Montgomery –Åsberg Depression Rating Scale; [7] Young Mania Rating Scale; [8] Information missing for 2 subjects; [9] Information missing for 9 subjects

Subjects with bipolar disorder showed higher CSF levels of kynurenine, KYNA, PIC and also higher CSF kynurenine/tryptophan and PIC/QUIN ratio compared to healthy controls. CSF levels of tryptophan did not differ between patients with bipolar disorder and healthy controls This was an indication that kynurenine pathway activation is not substrate driven. In addition, this is supported by other studies showing that TDO2 is more expressed (Miller et al., 2004) and KMO is less expressed in patients with bipolar disorder (Lavebratt et al., 2014a). Increased CSF KYNA levels have been found in this cohort of patients with bipolar disorder is in line with our previous finding (Olsson et al., 2010; Olsson et al., 2012; Lavebratt et al., 2014; Sellgren et al., 2016; Sellgren et al., 2019). CSF sampling in the current study was done seven years after the CSF sampling in the previous studies from the same cohort. In addition, PIC and ratio PIC/QUIN that were found to be elevated in CSF of patients with bipolar might be because of a possible activated immune system in the brain of those subjects, resulting in an activated kynurenine pathway, since IFN- γ, IL-1 β as well as IL-8, that are increased in those patients with bipolar, are known to induce both IDO and TDO2 (Söderlund et al., 2011; Isgren

et al., 2015; Isgren et al., 2017). All in all, the kynurenine pathway is activated in bipolar disorder and the activation is not substrate driven. It would be interesting to explore what would happen in case of bipolar patients undergo a diet of high tryptophan, having evidence that increased tryptophan intake in the diet can affect depression and mood state scores in healthy participants, resulting in improved mood states and less depressive symptoms (Lindseth et al., 2015).

4.2.3 Decreased levels of CSF PIC in patients with bipolar disorder with suicidal behavior

We further investigated if CSF levels of kynurenine metabolites were associated with suicidal behavior in bipolar patients. We found that CSF levels of tryptophan were higher ($P = 0.03$) while CSF levels of PIC ($P = 0.048$) and the ratio kynurenine/tryptophan ($\beta = -0.08$, $P = 0.04$) were lower in patients with bipolar disorder with a history of suicidal behavior compared to patients without any history of suicidal behavior. No differences in CSF kynurenine, KYNA, QUIN, or the PIC/QUIN ratio levels was found between the groups.

In our previous studies, we showed suicide attempters have reduced concentration of PIC in the CSF (Brundin et al., 2016) and remained reduced up to two years after. CSF QUIN levels were found to be elevated when subjects attempt suicide and have returned to normal six months later (Erhardt et al., 2013; Bay-Richter et al., 2015). Here we show that the concentration of PIC in CSF was reduced in bipolar patients who had a history of suicidal behavior compared to bipolar patients who did not have such a history. The QUIN levels in CSF did not differ in these sub-groups, a finding supporting the hypothesis that QUIN is only elevated at the time of the attempt. Importantly, in this study lumbar puncture was performed between one and seven years after the suicidal attempt.

Studies have shown that a combination of PIC and the mineral chromium called chromium picolinate helps subjects with atypical depression (Davidson et al., 2003; Docherty et al., 2005). Although the aim has not been to raise PIC levels, the metabolite´s subsequent elevation may help to partly clarify why the subjects´ symptoms improved. Low levels of PIC have also been reported in the plasma of subjects with unipolar depression, ETC treatment leads to elevated levels of PIC (Aarsland et al., 2019; Ryan et al., 2020). In addition, Radix Bupleuri-Radix Paeoniae Alba (RB-RPA), which is an herbal mix used for depression treatment, increases the bioavailability of PIC (Chen et al., 2021).

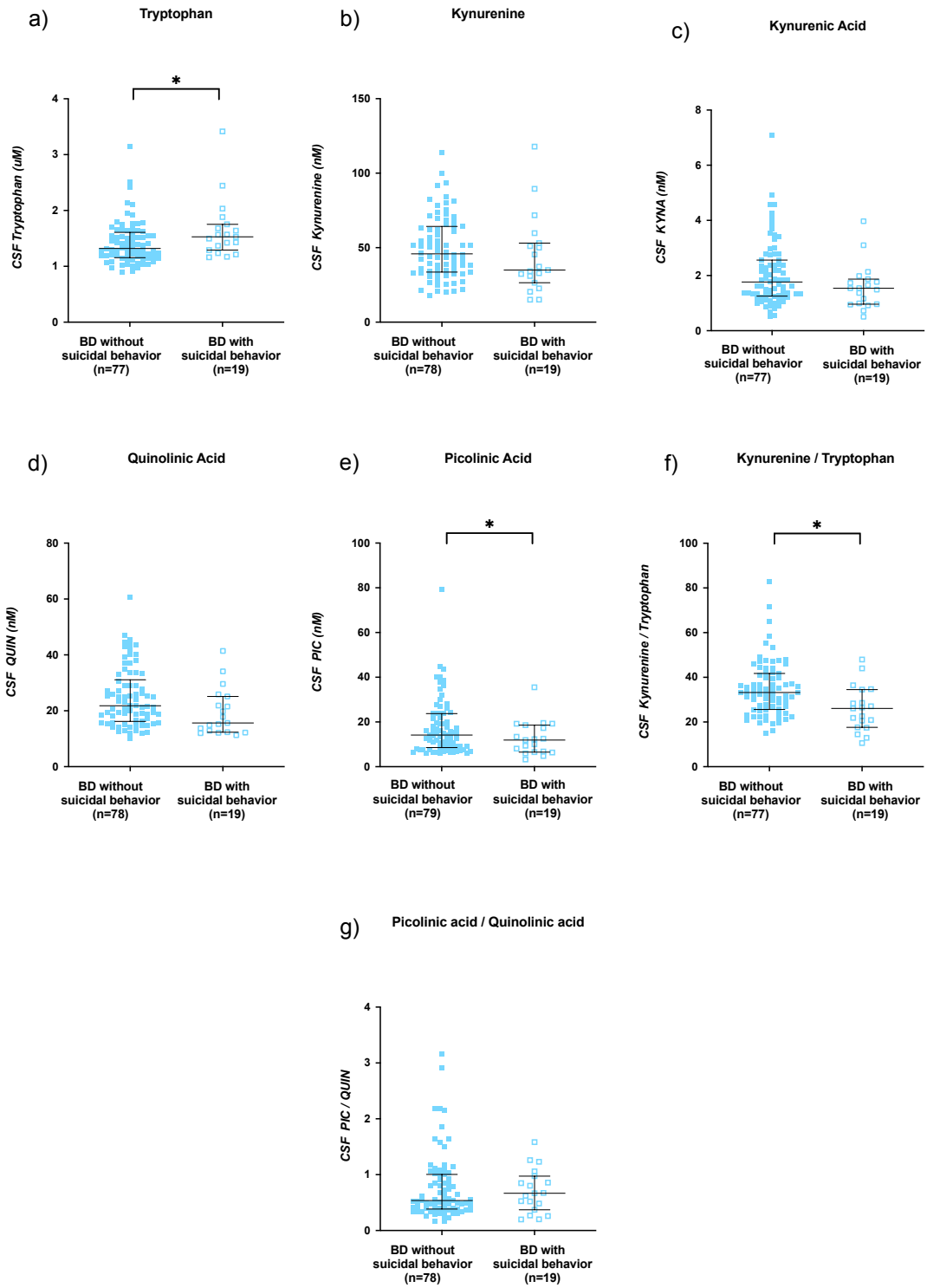

Figure 5. Cerebrospinal fluid levels of kynurenines in patients with bipolar disorder without and with suicidal behavior.

CSF levels of a) tryptophan, b) kynurenine, c) KYNA, d) QUIN, e) PIC, f) ratio kynurenine/tryptophan, g) ratio PIC/QUIN in patients with bipolar disorder that did not have suicidal behavior and the ones that had suicidal behavior. Each point represents the concentration of a single CSF sample, the horizontal lines represent the median for each group, and the bars represent IQR, Mann-Whitney, Logistic regression, ∗P ≤0.05.

4.2.4 Associations between pharmacological treatment and kynurenine metabolites in CSF of patients with bipolar disorder

The most commonly prescribed pharmacological treatments for patients with bipolar disorder are antidepressant drugs, antipsychotic drugs, lithium, lamotrigine, and valproate. Here, we investigated if different pharmacological treatments affected the levels of kynurenine metabolites in the CSF of patients with bipolar disorder.

Antidepressant drugs

Patients with bipolar disorder that received antidepressant drug treatment (n=42) had elevated CSF levels of kynurenine, KYNA, and higher kynurenine / tryptophan ratio (kynurenine: β = 0.3, P = 0.02; KYNA: β = 0.92, P = 0.001; kynurenine/tryptophan: β = 0.06, P = 0.02) than patients that did not receive antidepressant drug treatment (n=59). We did not find any differences for the other metabolites measured (tryptophan: P = 0.53; QUIN: β= 0.02, P = 0.45; PIC: P = 0.64; ratio PIC/QUIN: β= 0.33, P = 0.39) (Figure 6).

Antipsychotic drugs

Antipsychotic drugs did not affect the CSF levels of any kynurenine pathway metabolites (tryptophan: P = 0.59; kynurenine: β= −0.009, P = 0.45; KYNA: β = 0.0001, P = 1.0; QUIN: β = 0.32, P = 0.20; PIC: P = 0.29; ratio kynurenine/ tryptophan: β = 0.008, P = 0.71; ratio PIC/QUIN: β = −0.43, P = 0.32). 18 patients with bipolar disorder were treated with antipsychotics, while 83 were not.

Lamotrigine

When we compared patients with bipolar disorder who used lamotrigine (n=14) to those who did not (n=87), we found that only kynurenine was elevated in the ones that used lamotrigine (β = 0.04, P = 0.03). The other kynurenine metabolites showed no differences (tryptophan: P = 0.07; KYNA: β = 0.40, P = 0.13; PIC: P = 0.08; QUIN: β = − 0.02, P = 0.64; ratio kynurenine/ tryptophan: β = 0.03, P = 0.21; ratio PIC/QUIN: β= 0.84, P = 0.09).

Lithium

CSF levels of kynurenine pathway metabolites between patients with bipolar disorder who used lithium (n=54) and those who did not (n=47) did not differ (tryptophan: P = 0.15; kynurenine: β = 0.02, P = 0.16; KYNA: β= 0.17, P = 0.38; QUIN: β= 0.01, P = 0.54; PIC: P = 0.17; ratio kynurenine/tryptophan: β = 0.02, P = 0.25; ratio PIC/QUIN: β = −0.74, P = 0.07).

Valproate

There were no differences in CSF kynurenine pathway metabolites between patients with bipolar disorder who used valproate (n=9) and those who did not (n=92) (tryptophan: P = 0.2; kynurenine: β = −0.006, P = 0.81; KYNA: β = −0.08, P = 0.87; QUIN: β = −0.18, P = 0.09; PIC: β = −0.12, P = 0.82; ratio PIC/QUIN: β = 0.91, P = 0.13).

The associations we found between antidepressant treatment and elevated kynurenine and KYNA levels in CSF, and also between treatment with lamotrigine and elevated kynurenine levels in CSF, are supported by rodent studies. When rodents were treated with antidepressants that selectively inhibit serotonin reuptake, reduced KMO gene expression, elevated astrocytic KAT1 and KAT2 gene expression as well as lower QUIN brain levels were seen (Kocki et al., 2012; Eskelund et al., 2017). Thus, antidepressant therapeutic efficacy might be enhanced by directing the kynurenine pathway's activity toward the development of the NMDA receptor antagonist KYNA rather than QUIN.

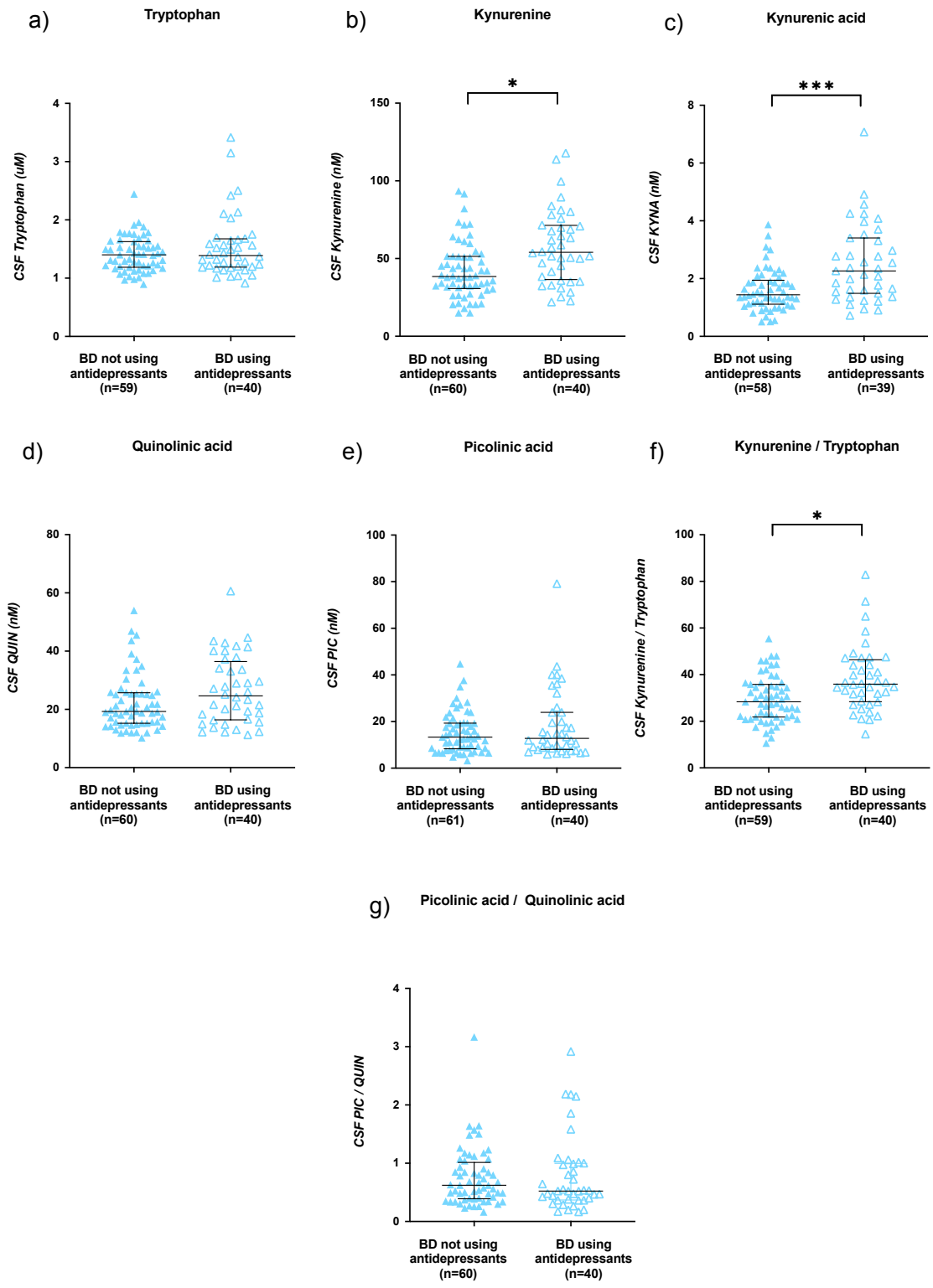

Figure 6. Cerebrospinal fluid levels of kynurenines in patients with bipolar disorder not using and using antidepressant drugs.

CSF levels of a) tryptophan, b) kynurenine, c) KYNA, d) QUIN e) PIC f) ratio kynurenine/tryptophan, g) ratio PIC/QUIN in subjects with bipolar disorder not using antidepressants and using antidepressants. Each point represents the concentration of a single CSF sample, the horizontal lines represent the median for each group, and the bars represent IQR, Mann-Whitney, Logistic regression, ∗P ≤0.05; ∗∗∗P ≤0.001.

4.2.5 Associations between CSF kynurenine metabolites and symptoms

MADRS and YMRS scores did not correlate with any of the metabolites in the CSF of patients with bipolar disorder. Moreover, the number of depressed, manic, hypomanic, or mixed episodes did not correlate to any of the kynurenine metabolites. We found a tendency towards a negative correlation between the number of manic episodes and the CSF KYNA concentration ($r = -0.21$, $P = 0.06$).

Age of patients with bipolar disoder	Number of mood episodes (median, IQR)				Duration of illness in years (median, IQR)
	Depressive	Hypomanic	Manic	Mixed	
<35 (n=26)	8 (3-12.5)	2 (0.5-5)	1 (1-3.5)	0 (0-0)	17 (13.5-19)
36-50 (n=41)	6 (3-17)	6 (2-29)	1 (1-4.25)	0 (0-1)	22 (19-25)
51< (n=34)	7 (3-14)	2.5 (0.25-8.75)	1 (0-4)	0 (0-0)	35.5 (24.25-45.25)

Table 13. Summary of mood episode history of patients with bipolar and their duration of illness

4.2.6 Common genetic variation in ACMSD and suicidal behavior

In genotyped patients with bipolar disorder (n=99) we explored the effects of ACMSD variants (tag SNPs) on CSF QUIN, PIC, and the PIC/QUIN ratio. The SNPs rs6722883 associated negatively with the CSF PIC/QUIN ratio of bipolar disorder patients with a history of suicidal behavior (n=19) after controlling for age, sex, and *post-hoc* Bonferroni correction: ($\beta = -1.77$; $P = 0.001$). Thus, the negative association found here between the tag SNPs rs10928521 and rs6722883 and the ratio PIC/QUIN is supporting the results from our previous study (Brundin et al., 2016) showing that specific polymorphisms of the ACMSD gene associate with higher levels of QUIN and lower levels of PIC.

4.3 Kynurenine metabolites in the CSF and plasma of healthy subjects following physical exercise (Paper III, IV)

4.3.1 Sprint interval training

Participants of the study were divided into old subjects 64.3±5.7 years old (mean±SD) and young subjects 24±3.2 years old (mean±SD). Plasma was collected before SIE (baseline), one and 24 hours after the SIE was performed. Baseline levels of kynurenine metabolites did not differ between young and old participants (see Table14).

Compound	Young baseline	Old baseline	P values*
Tryptophan	43.8 μM ± 4	43.7 μM ± 4.3	0.97
Kynurenine	2.5 μM ± 0.2	3.0 μM ± 0.3	0.25
KYNA	49.5 nM ± 5.4	56.2 nM ± 15.1	0.63
3HK	33.7 nM ± 4.4	43.6 nM ± 8.9	0.35
QUIN	422.0 nM ± 61.4	404.8 nM ± 62.1	0.84
PIC	58.4 nM ± 7.4	92.1 nM ± 29.1	>0.99
NAA	127 nM ± 18.8	97.6 nM ±9.6	0.25

Table 14. Baseline levels of kynurenine metabolites in young and old subjects
*Mann-Whitney U-test (mean ± SEM)

One bout of SIE did not affect any of the metabolites in the plasma of young subjects at any of the timepoint tested (see Figure 3). In old subjects, plasma kynurenine levels were found to be elevated one hour after the bout of SIE (P = 0.03) (Figure 3d) and plasma KYNA levels significantly elevated 24 hours after (P = 0.02) (Figure 7d). The other kynurenine metabolites were not affected by SIE.

Kynurenine and KYNA changes seen only in old subjects might be due to the higher relative SIE intensity since the SIE intervention in this study was related to body weight, and the muscle mass is known to decrease by age (Heo et al., 2018).

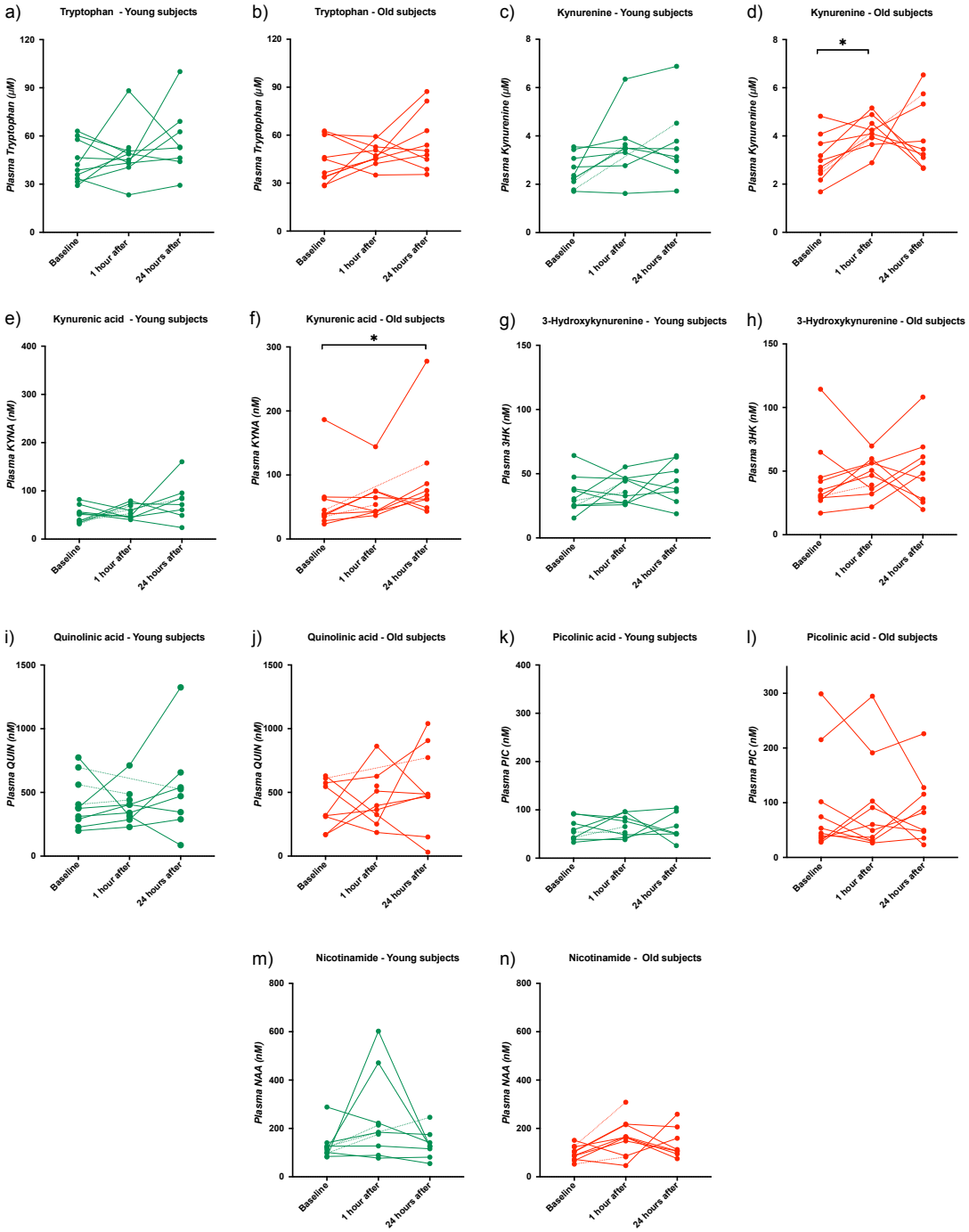

Figure 7. Plasma concentrations of kynurenines at baseline, 1 hour and 24 hours after SIE, in young (green) subjects and in old (red) subjects.

Each dot represents the concentration of respective metabolite in nM (µM for tryptophan and kynurenine) of a single plasma sample. A dotted line is used when a timepoint data is missing for the subject. * P<0.05; **P<0.01, Kruskal-Wallis post-hoc Dunn's.

4.3.2 Aerobic exercise

Tryptophan, kynurenine, KYNA, 3-HK, PIC and QUIN were analyzed in the CSF and plasma of 27 subjects. Subjects were sub-grouped based on their exercise habits. Thus, 14 subjects were placed to the acute exercise group and 13 allocated to the training exercise group. All kynurenine metabolites were successfully detected in 26 subjects. Both baseline and follow-up exercise CSF from one subject had a coefficient of variation more than 10% in the detection of duplicates for PIC, so it was excluded from the statistical analyses. A summary of participants characteristics is presented in Table 15.

	Total study sample (n=27)	Acute group (n=14)	Training group (n=13)
Sex (male/female)	12/15	6/8	6/7
Age (years) [a]	28.7±7.7, 22–52	28.1±8.7, 22–52	29.3±6.8, 22–45
Body Mass Index (kg/m^2) [a]	22.6±2.6, 18.9–29.6	22.9±2.7, 19.6–29.6	22.2±2.5, 18.9–28.0
Smokers	0	0	0
History of or active substance use disorder	0	0	0
Continous medication	0	0	0
Exercise habits [b] (low, moderate, high)	Low (n=6) Moderate (n=7) High (n=14)	High (n=14)	Low (n=6) Moderate (n=7)

Table 15. Characteristics of the study population.

Individuals that participated in the study were allocated in two groups based on their exercise habits. [a] Mean ± SD., range. [b] Self-reported measure of recent exercise activity.

CSF concentrations of KYNA, 3HK and PIC levels were increased after the acute exercise paradigm, while tryptophan and kynurenine remained unchanged. Moreover, the CSF ratio of kynurenine/tryptophan increased in the training group. On the contrary, tryptophan and kynurenine strongly decreased after acute exercise, whereas KYNA, 3-HK, QUIN and PIC in plasma did not change in any of the groups following exercise (see Figure 8).

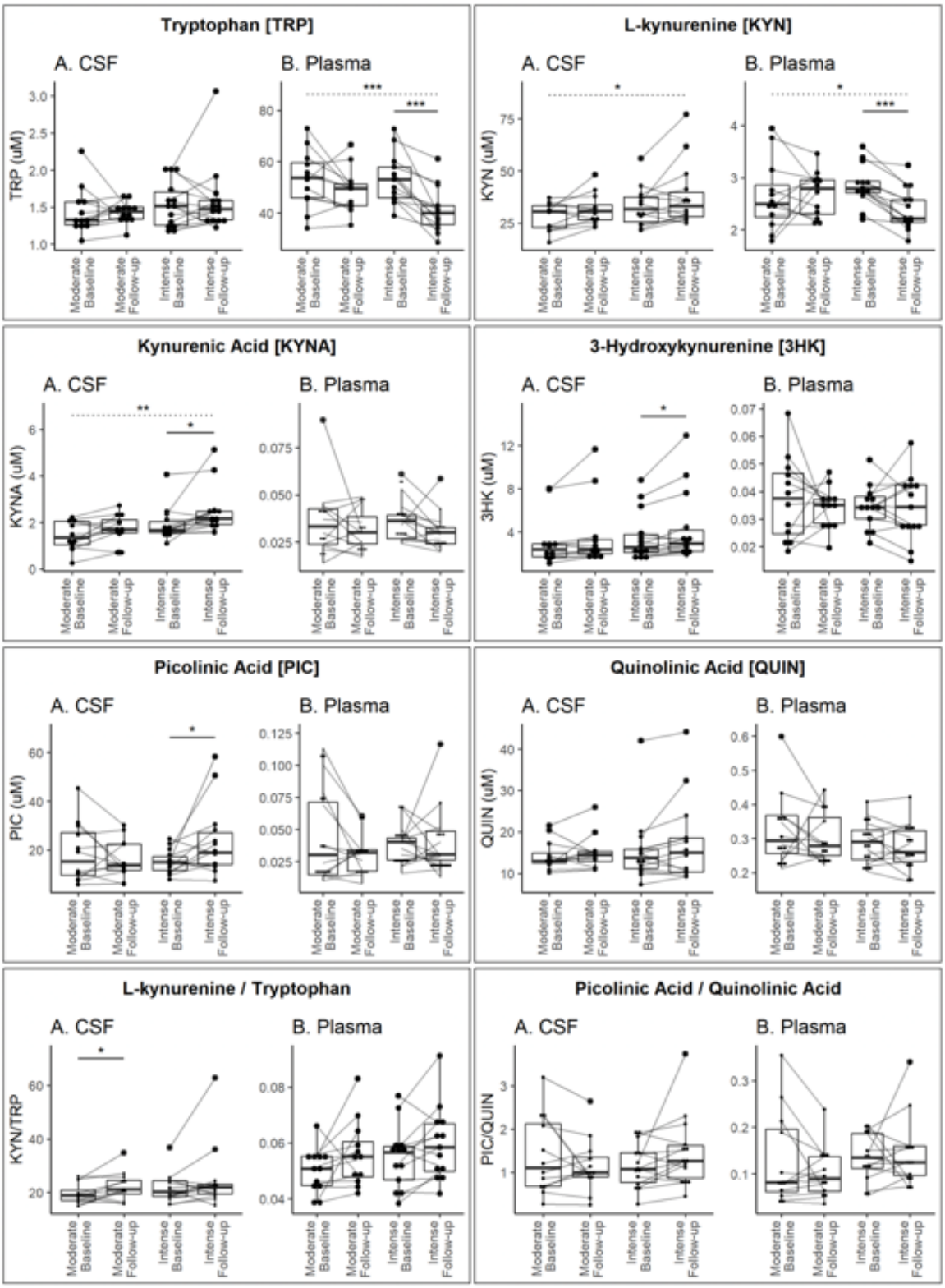

Figure 8. Cerebrospinal fluid and plasma concentration of kynurenine metabolites stratified by exercise intervention.

Box and dot plots illustrate metabolite levels (μM) in a) cerebrospinal fluid (CSF) and b) plasma among paired samples (line) baseline and follow-up moderate or high intensity anaerobic exercise. Significance was determined for all samples (dotted bar) and when stratified by intensity (solid bar). * P<0.05; **P<0.01 and *** P< 0.005.

Correlations between CSF and plasma concentrations of kynurenine metabolites were in general weak. Interestingly though, PIC and showed a strong correlation between CSF and plasma both at baseline and follow up (see Figure 9).

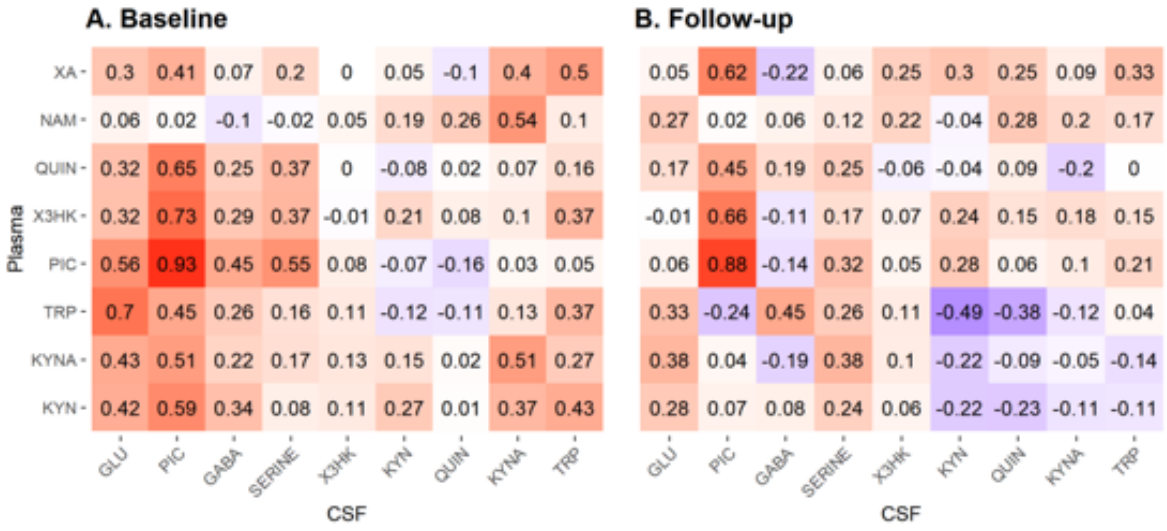

Figure 9. Heatmaps showing the correlation between metabolites analyzed in cerebrospinal fluid with those in plasma.

Pearson correlation coefficients are listed and color-coded for each cell. (A) baseline; (B) follow-up.

4.3.3 Neurotransmitters in the CSF of healthy subjects

Concentrations of the neurotransmitters glutamate and GABA analyzed at the baseline and follow up-intervention did not change in either groups. CSF serine levels were though found to be elevated following acute exercise intervention (Figure 10).

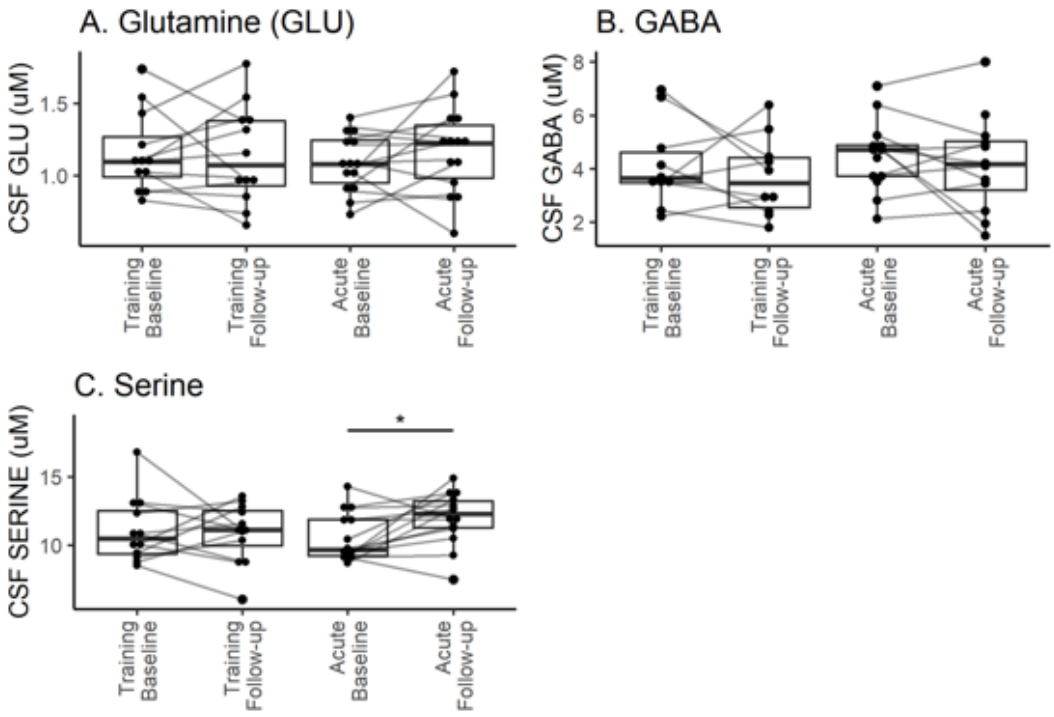

Figure 10. Box and dot plots representing (A) glutamine, (B) GABA and (C) serine levels (µM) in cerebrospinal fluid (CSF) among paired samples (line) at baseline and follow-up training or acute exercise.

* P < 0.05, ** P < 0.01, and *** P < 0.005.

4.3.4 Immune markers in the CSF and plasma of healthy subjects

PEA was used to investigate a panel of 92 inflammation-related proteins in both CSF and plasma. Only proteins with a paired call rate > 70% were used for further analysis (CSF n = 47; plasma n = 68). One marker (BDNF) was excluded due to issues with assay reliability. Three out of the 47 proteins were found to be upregulated in the CSF of subjects performing acute physical exercise. On the contrary, no changes were found after the training exercise intervention (Figure 11 A, B). After the acute exercise program 12 out of 68 proteins were upregulated in plasma, although, again, no changes were found after the training exercise intervention (Figure 11 C, D). We found only minor differences in baseline protein levels in plasma and CSF in both groups by using cross-sectional analysis (Figure 11 E, F).

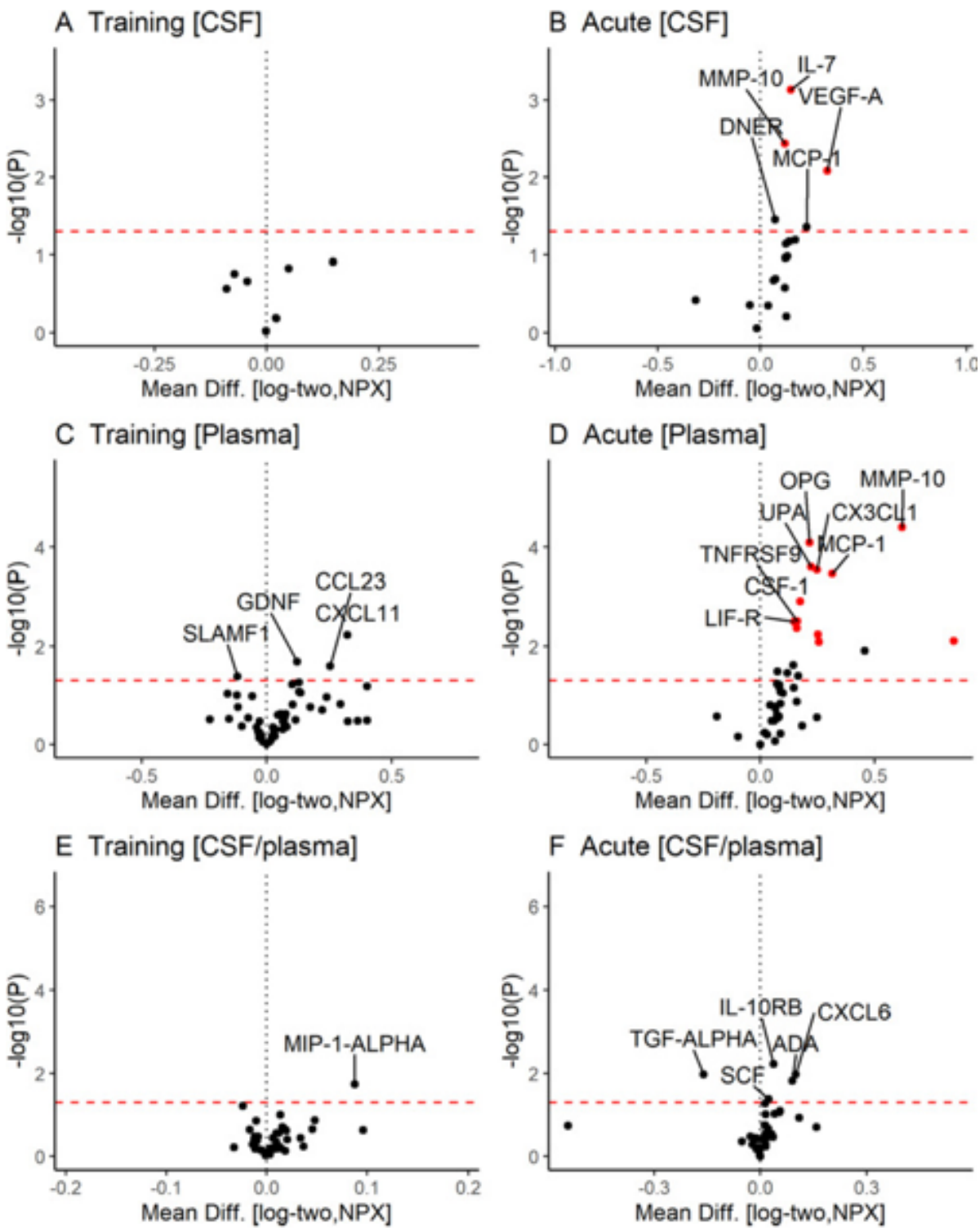

Figure 11. Changes in the immune marker profile of cerebrospinal fluid (CSF) and plasma after training and acute intervention exercise.

Volcano plots show the mean difference and significance (P) from paired Student t-tests comparing baseline and follow-up levels of immune markers in CSF (A,B), plasma (C,D), and the ratio of CSF/plasma (E,F), after intervention with training (left) or acute (right) intensity exercise. The red dashed line indicates an exploratory cutoff of P=0.05 and associations with P_{FDR} <0.05 are presented as red dots.

In detail, CSF vascular endothelial growth factor (VEGF) was found to be upregulated after the acute exercise. In line with this, one study showed that VEGF levels in the blood rise in proportion to the exercise intensity (Marquez et al., 2015). There was only one protein upregulated in both plasma and CSF after acute exercise and it was matrix metalloproteinase (MMP)-10. Upregulation of MMP-2 and MMP-9 has previously been associated with the exercise of high intensity (Carmeli et al., 2005). We found that CSF IL-7, and plasma transforming growth factor-alpha, delta, leukemia inhibitory factor receptor, and notch-like epidermal growth factor, were all increased after the acute exercise, however, none of those had previously been shown to have an association with physical exercise. In addition, some cytokines and chemokines were increased in plasma after acute exercise, however, that was not the case for CSF.

4.3.5 Relation CSF-plasma of kynurenine metabolites and immune markers in healthy subjects

To investigate if changes in immune markers in plasma and CSF after the physical exercise correlated with changes in kynurenine metabolites, two exercise groups were combined. We found that in the CSF of subjects after exercise kynurenine, QUIN, kynurenine/tryptophan ratio, and less the PIC/QUIN ratio correlated with an aberrant profile of immune markers. We also found that in plasma of subjects after exercise kynurenine and PIC, and less KYNA associated with alterations in the profile of immune markers (Figure 12).

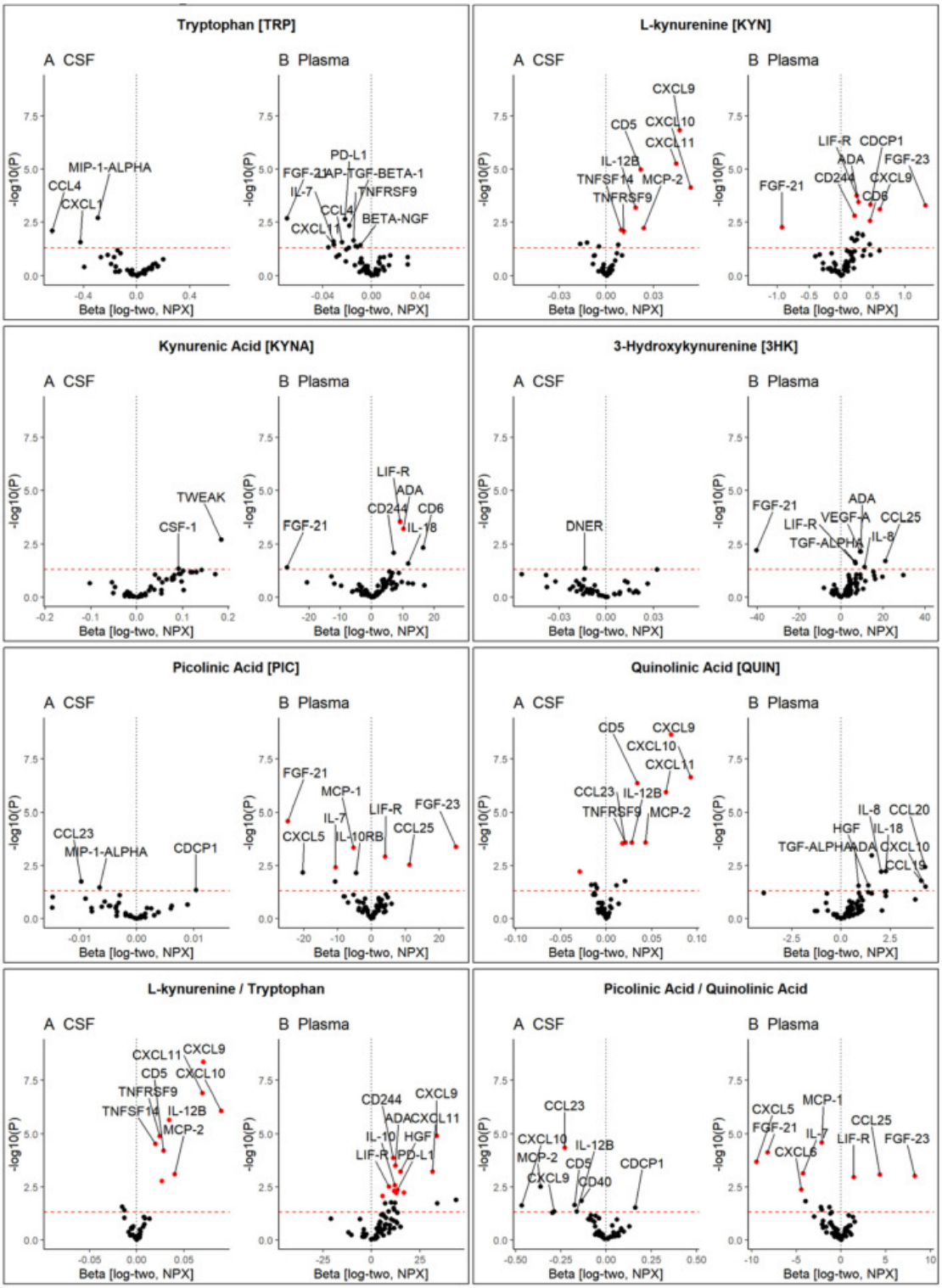

Figure 12. Correlation between levels of immune related protein and kynurenines

Volcano plots summarize the effect size (B) and significance (P) from linear regression analyses between metabolite and protein levels adjusted for age at baseline, sex, intervention, and sample handling variability. Red dashed line indicates an exploratory cutoff of P=0.05 and associations with P_{FDR} <0.05 are presented as red dots.

5 GENERAL DISCUSSION

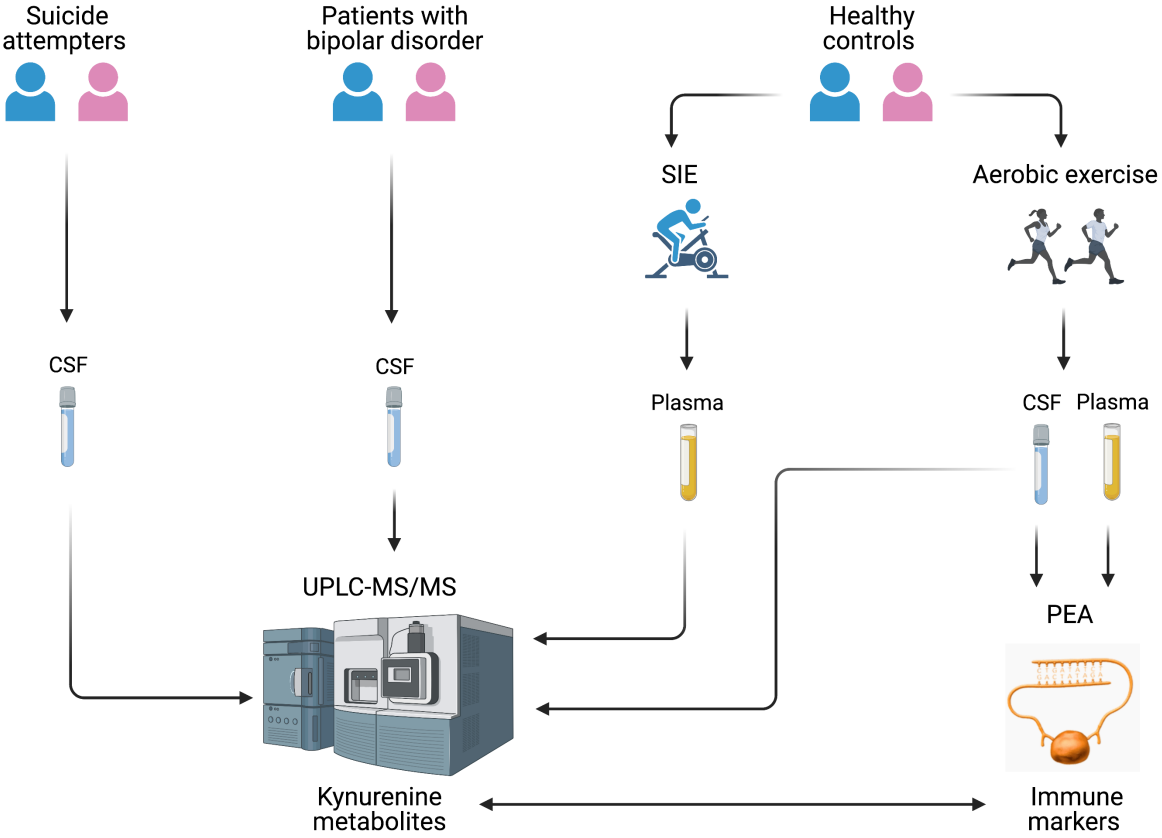

Figure 13. An overview schematic illustration of the workflow of the thesis

(created with Biorender.com)

In the present thesis, we have developed a robust, sensitive, and reproducible method to detect and simultaneously quantify ten kynurenine metabolites in human CSF and plasma using UPLC-MS/MS. Importantly, our method provides clear chromatographic separation of the two isomers PIC and NA. Thus, our newly developed method has several advantages compared to other methods used for analyzing kynurenines. In addition, the simultaneous analysis of such many metabolites allows for a minimum volume.

In order to use kynurenine pathway metabolites as biomarkers on a daily basis in the clinic, it is of essential importance to know how stable the metabolites are. Our results show that, in general, the metabolites are very stable both in CSF and plasma. They are thus, stable at room temperature for up to 4 hours and they do not degrade following multiple freeze-thawing. Long-

term storage (up to 24 hours) at room temperature might though affect CSF 3-HK concentrations and plasma QUIN levels. In addition, data presented in this thesis suggest that plasma 3-HANA and XA concentration might increase following 2-3 hours of being stored at room temperature.

Biomarkers are becoming increasingly important in all fields of clinical practice, as they may be used to diagnose, monitor, or predict illness at any stage during patient care. This is of particular importance in psychiatry (Le-Niculescu et al., 2021), where the diagnose is solely based on symptoms. Biomarkers may thus provide an objective, measurable way to characterize the disease. Several of the kynurenine pathway metabolites have been suggested as putative future biomarkers for psychiatric disorders. Thus, previous studies have shown that QUIN is increased, and PIC is decreased in the CSF at the time of suicide attempt. While CSF QUIN levels return to normal levels within 6 months after the attempt, CSF PIC levels remain low throughout a period of two years after the suicide attempt (Erhardt et al., 2013; Brundin et al., 2016). Low CSF PIC is thus suggested to indicate vulnerability for suicidal behavior (Brundin et al., 2016). In the present thesis, we confirm a low PIC/QUIN ratio in the CSF of suicide attempters compared to healthy controls. Interestingly, we also discovered that CSF PIC levels were lower in a sub-group of patients with bipolar disorder with a history of suicidal behavior, compared to bipolar disorder patients that had not experienced suicidal behavior. In line with the hypothesis that CSF QUIN levels are only elevated at the time of a suicide attempt, the CSF QUIN levels were similar to the levels in healthy controls. In the bipolar disorder cohort, we further confirmed that CSF KYNA levels are increased compared to healthy controls.

Previous studies suggest that the imbalance between QUIN and PIC might be related to lower expression and/or lower activity of the enzyme ACMSD in suicide attempters (Brundin et al., 2016). In the present thesis, we also found that genetic variations in the ACMSD gene contribute to this imbalance. As such, the enzyme ACMSD might be a potential therapeutic target for suicidal behavior. When developing a biomarker, it is of importance to have control of confounding factors. Indeed, several studies have shown that physical exercise affects the levels of peripheral kynurenine pathway metabolites. However, there is limited information on how physical exercise affects brain levels of the metabolites and if central levels correlate to levels in the periphery. In the present thesis, we present data on how SIE affects plasma levels of kynurenine pathway metabolites in different age groups of healthy subjects. In addition, we show how aerobic exercise affects central and peripheral levels of these metabolites in healthy subjects. Thus, one bout of SIE increased plasma kynurenine and KYNA levels in old, but not

young, healthy subjects. The disparity between young and old subjects is believed to be related to skeletal muscle physiology (Westbrook et al., 2020). Thus, muscle mass, known to decrease with age (Heo et al., 2018), indicates that the relative training intensity was higher in older subjects. The different effects may also be related to age-related variations in immune activity (Walston et al., 2002; Varadhan et al., 2014). Different intensity of aerobic exercise was also found to affect kynurenine pathway metabolites in both plasma and CSF. In addition, we found minor changes in immune markers, and in line with data showing immune-induced activation of the kynurenine pathway, we found that several of the immune markers correlated with the concentrations of kynurenine pathway metabolites. Generally, though, we found no correlation between plasma and CSF levels of immune markers or between kynurenine pathway metabolites in the CSF and plasma. The only exception was PIC, which showed a high correlation between plasma and CSF, both before and after exercise. This is interesting in view of the fact that low plasma PIC levels are also found in suicide attempters (Brundin et al., 2016).

According to FDA, there are seven categories of biomarkers; risk, diagnostic, prognostic, predictive, pharmacodynamic, safety, and monitoring biomarkers. Analyzing the feasibility of using PIC as a biomarker for suicide and/or suicidal behavior in patients with bipolar disorder – diagnostic, risk, prognostic, and predictive biomarkers might be the case. Using a diagnostic biomarker, first suicide should be classified as a novel diagnosis and second a cut-off value must be set. Also using a risk biomarker, suicide should be considered a diagnosis itself. PIC as a prognostic biomarker could be the case of suicidal behavior as a preexisting condition in patients with bipolar disorder. In such cases, the basal level for the biomarker should be established. In addition, prognostic biomarkers should be specific for the present diagnose, i.e., it should be specific for bipolar disorder and no other psychiatric diagnoses. Predictive biomarkers instead are used to distinguish subjects who have the highest chance of responding positively to a given intervention. In conclusion, our data support the hypothesis that PIC could be developed into a potential biomarker indicating vulnerability for suicide behavior.

In conclusion, by using a newly developed method for analyzing kynurenine pathway metabolites, we here confirm an activated kynurenine pathway in the brain of bipolar disorder patients. We also find support for the hypothesis that low PIC levels could be a marker indicating vulnerability for suicidal behavior and we show that exercise influences both central and peripheral levels of kynurenine pathway metabolites.

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